

Laser facility under scrutiny

Sudden and unexpected cost overruns at the US National Ignition Facility have once again called into question the ability of the Department of Energy to build major scientific facilities on time and on budget.

When Bill Richardson, a US energy secretary who has spent most of 1999 mired in the repercussions of the Chinese spying scandal, visited the Lawrence Livermore nuclear weapons laboratory in June, it seemed that for once he had something to celebrate. The National Ignition Facility (NIF), a \$1.2 billion laser device currently under construction at Livermore to study inertial confinement fusion, was on schedule and on budget (see *Nature* 399, 622; 1999).

It's therefore little wonder that Richardson blew his top when he found out at the beginning of this month that both propositions look dubious, to the tune of two years and several hundred million dollars, respectively, and that this has been common knowledge inside the laboratory — and, perhaps, in his department — at least since April (see page 201).

It is not yet entirely clear what issues are dogging the NIF. The Department of Energy (DoE) says that the main problem is that Livermore's original plan, for its own scientists and engineers to assemble the facility's 192 giant lasers on site, would not provide a sufficiently dust-free environment to ensure the reliable operation of the powerful but sensitive laser optics. Bringing in an outside contractor with experience of clean rooms to do this properly will, according to the DoE, delay the project and rapidly inflate its cost.

But other technical problems lurk in the background. Richardson is confounded to discover that the most up-to-date and reliable information on the status of the project has been arriving from watchdog groups outside the laboratory, rather than from his own officials. Taxpayers and the Congress are entitled to share his exasperation.

From the start, the NIF, the largest component of the energy department's stockpile stewardship programme for nuclear weapons, has looked tricky to execute. It is really a cut-price version of an abandoned \$3 billion plan for a laser microfusion facility, and some experts doubt that it will achieve the fusion ignition promised in its name.

The project has been justified in terms of the stockpile stewardship mission, as ensuring the safety and reliability of US nuclear weapons. But the safety issue is a complete red herring, and the reliability argument is subject to fierce debate, even inside Livermore (see *Nature* 386, 645–647; 1997). The NIF's real function, in fact, is to serve as a sandbox for US weapons scientists until nuclear weapons development and testing can resume.

Beyond that unhappy circumstance, the main value of the NIF is that it could help to establish the potential of inertial confinement fusion as an energy source. But the energy interest has always taken second place, as the project is paid for from the nuclear weapons budget. Proposals to establish a new nuclear weapons agency inside the DoE, together with the retrenchment of the NIF to deal with the cost overruns, threaten to further marginalize energy research on the facility.

The full extent of the NIF's troubles remain unclear, and various investigations will soon be under way. They will no doubt focus on how a \$1.2 billion project could be as much as \$300 million over budget before the alarm is sounded; hopefully, the energy department and the Livermore laboratory will come up with more convincing explanations than those offered so far. ■

A tool of the trade

The French government must reveal the calculations behind its decision to drop plans for its own synchrotron.

British scientists arguing over where to build Diamond, the country's £175 million (US\$280 million) next-generation synchrotron (see page 197), should spare a thought for their French colleagues. A decade of plans for a similar French machine, Soleil, were dashed last month by Claude Allègre, the science minister, who decided instead to invest one-third of the cost of Soleil in Diamond itself.

As with all large science facilities, the decision on the UK machine will ultimately involve a variety of factors, including both the economic and the scientific. The arguments for and against are, predictably, being vigorously defended by the various lobbies. The best that can be hoped for is that the interests of all the researchers whom the machine is intended to serve are fully and honestly articulated by the parties, and taken into account seriously by the government.

In such important issues, a government has a duty to explain clearly the basis on which a decision has been reached. So far, this duty seems to have been bypassed by Allègre's ministry, which, apart from muttering about the need for greater European integration, has not presented in detail a convincing explanation for abandoning Soleil. Nor has Allègre improved his image among working scientists by making misleading statements to the French National Assembly unfairly discrediting the Soleil project.

Synchrotrons are not 'big science' projects; rather, they are tools shared by scientists in disciplines from molecular biology to materials science who need to know about the structure of matter. This complicates the equation about user needs. A number of independent reports published in France and at the European level over the past few years have concluded that such needs in Britain and France are more than sufficient to justify two machines. Allègre's decision is based on a report by one of his advisers that has never been made public. Perhaps his ministry knows something that others do not; but surely this self-declared champion of peer review and independent evaluation should expose the evidence to critical appraisal.

Ministry officials admit, for example, that the FF350 million (US\$56 million) investment in Diamond will at most meet a third of French user needs, the rest being met by the ageing LURE facilities near Paris, and by hiring beam-lines on machines in other countries. Not only trade unions, but also more independent French personalities, question whether the figures add up, and whether building Soleil might not turn out to be both cheaper and more efficient in the long run. For the moment, the only proposal that might calm both French and UK scientists would be to build a much bigger synchrotron halfway through the Channel Tunnel. ■





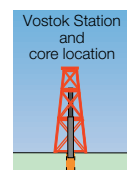
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Staff accuse bosses of secrecy over British synchrotron plans...

London

Tensions are running high in Britain over whether its planned next-generation synchrotron source, Diamond, will be built at the Daresbury Laboratory, outside Manchester in the north of England, or at the Rutherford Appleton Laboratory (RAL), south of Oxford.

Scientific and technical staff at Daresbury, who operate the current UK Synchrotron Radiation Source and have worked on the £175 million (\$280 million) Diamond project for many years, say senior management has kept them in the dark over discussions about possibly siting the facility at the RAL.

Both sites are run by the Central Laboratory of the Research Councils (CLRC) — a third suggestion of a 'green field' site is said to have been ruled out. Diamond is a joint venture between the British government, the Wellcome Trust and France.

A decision in favour of the RAL would be a blow to Daresbury staff, whose union representatives last week met the science minister, David Sainsbury, to put the scientific and economic case for housing the new source near Manchester. Sainsbury has also heard from local members of parliament, trade unions and the regional development agency.

They say that losing Diamond would be an economic setback for the area, as Daresbury employs 500 of the region's 800 government scientists. Union representatives from both laboratories support Daresbury's bid and claim support from staff at both sites, expressing concern that, without Diamond, the CLRC might close Daresbury.

Local MP Mike Hall points out that Daresbury has been supported by the international user community. As the expertise to run Diamond is currently at Daresbury, he says, moving the machine elsewhere would mean relocating staff, and capital costs would be £32 million higher.

The CLRC is keen to distance itself from the lobbying. "It's not our remit," said a spokeswoman. "We've been providing scientific and financial information." She says that the CLRC has no preferred site.

But Daresbury staff complain that senior



Moving south? Daresbury staff are surprised by the competition from the Rutherford Appleton.

CLRC staff have only recently allowed discussion over the site. Susan Smith, a union representative and scientist at Daresbury, says this separated Daresbury staff, as technical advisers, from senior CLRC management at the RAL, who were potential bidders. "We were told not to speak to anyone or contact anyone. Information from senior management to staff was stopped."

This has put Daresbury at a serious disadvantage, says Smith. When staff were told for the first time last month that the RAL was a contender, it came as "a complete shock".

It is unclear who is behind the move to site

Diamond at the RAL. Daresbury staff speak darkly of an "Oxford-Cambridge mafia", and there are rumours that the Wellcome Trust, which is providing most of the construction money (see *Nature* 394, 209; 1998), also favours this site.

Since announcing its contribution, the trust has discussed Diamond's site with the Office of Science and Technology (OST), and is said by some to prefer the Oxford location because it is closer to the UK's biomedical community in the south-east of England.

The trust, which declines to comment on the debate, has previously said that a decision on location would not be taken until potential users had been consulted "in collaboration with the research councils".

But little such consultation seems to have taken place. Mark Palmer, a member of the Medical Research Council's research management group, says the consultation on the use of Diamond by the biological sciences was intended to produce a technical report, and that the exercise "did not address location".

The Office of Science and Technology is said to have commissioned an independent site review. The decision on the site is expected to be announced in the next few weeks.

Natasha Loder and Karen Birmingham

... and French switch off in protest

Paris

French scientists, angered at last month's government decision to scrap plans to build a new synchrotron in France and instead join with Britain to build a new machine there, have protested by refusing to restart two synchrotron machines after the summer vacation.

The researchers are continuing to challenge the financial basis of the government's decision and the argument that the future synchrotron needs of French scientists can be adequately met by using the UK-based machine and renting beamlines elsewhere in Europe.

In early August, Claude Allègre, the minister for national education, research and technology, announced that the French government would give FF350 million (US\$56 million) towards Diamond, a 3-GeV synchrotron, to be built in Britain (see *Nature* 400, 489; 1999 and above). This ended hopes that the government would construct a 2.15-GeV source, Soleil, which had been in the planning stage since 1991.

The move has incensed scientists and some French politicians, who have lobbied for eight years to build Soleil to replace two ageing facilities at LURE (800 MeV and 1.85 GeV), south of Paris. "We are responding to

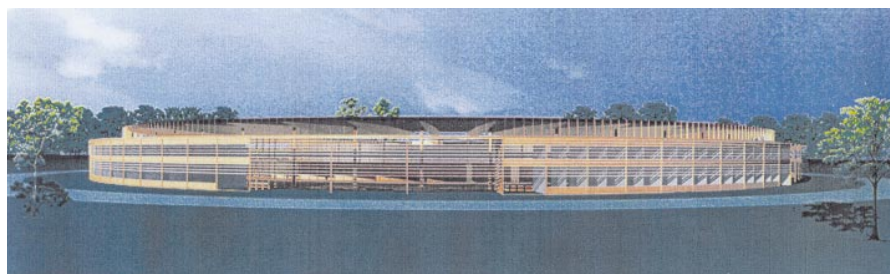
the minister, who, in making this decision, said that LURE doesn't serve a purpose," says Robert Comès, LURE's director. "If it doesn't serve a purpose, there's no reason to have it in use."

More than 300 scientists at LURE met last Monday and voted unanimously — with two abstentions — to leave the machines, which were shut down for August, turned off. Repeating their conviction that Soleil is essential for the future of French science, they agreed to refuse to collaborate in the Franco-British project, and demanded that Allègre reverse his decision.

However, Allègre, who has made European collaboration a cornerstone of his policy, shows no sign of relenting. According to Vincent Courtillot, director of research for the ministry, the government has been trying to trim costs on 'big science' facilities in order to inject more money into research at public laboratories.

"There is no doubt that synchrotron radiation is an important and necessary technique," says Courtillot. "The one thing we have been questioning is whether [Soleil] is really necessary, and whether we are prepared to give a large amount of money to it."

Courtillot admits that Diamond will fulfil only about one-third of French researchers' needs, but adds that the government is already negotiating to rent beamlines on BESSY II in Germany, SLS in Switzerland and Elettra in Italy.



Eclipsed: plans for the Soleil synchrotron facility have been dropped by the French government.

"This should give us two-thirds of Soleil," Courtillot says. He adds that the government plans to join with Britain, France, Germany and Italy in coordinating the development and operation of large facilities, among them synchrotron radiation and neutron sources. This is expected to take up to four years to set up.

Several experts, however, have challenged the ministry's strategy, arguing that it could end up costing more than Soleil, while meeting a substantially smaller fraction of French research needs than Courtillot estimates. For example, researchers say that Diamond will meet one-quarter of their needs, not one-third.

Many question why Soleil is being abandoned for a European project if that project doesn't offer substantially greater benefits. "We have to look at what everything is going to cost, and what the advantages and disadvantages are," says one expert close to the project. "The debate has

been very passionate, but there has been no rational discussion."

Soleil, projected to cost FF1 billion, was to have received FF675 million in regional aid. Scientists point out that the remainder is almost exactly what the government is pledging towards Diamond, and that this does not take into account the cost of renting future beamlines, the additional upkeep of LURE, or travel budgets.

Scientists have demanded that Allègre reveal the details behind his decision, and particularly that he publish a report he commissioned that was favourable to the British plan.

"It seems very curious that the minister made [this] decision without having any interactions with the scientists involved," says one expert.

The protest is likely to continue for a couple of weeks. About 400 scientists work at LURE, and 1,800 visit each year to use its facilities.

Heather McCabe

Obuchi vows to push university reforms in Japan

Tokyo

A plan by the Japanese government to transform the country's 99 national universities into semi-autonomous 'agencies' moved a step closer to reality last week when Keizo Obuchi, the prime minister, promised to enforce the reform if he is re-elected head of the ruling Liberal Democratic Party (LDP).

Obuchi stressed the importance of restructuring the universities in a statement on education policy issued as part of his re-election campaign. The plans are part of a broader effort to increase administrative efficiency by turning government-run organizations into semi-autonomous bodies (see *Nature* 389, 897; 1997).

Koichi Kato, Obuchi's main opponent in the election and a supporter of Japanese life-science research, also backs the agency plan as a step towards privatizing the universities. Kato has been critical of the current university system, particularly the restrictions of civil-service law which prohibit researchers from taking part in commercial activities.

Spurred on by this political pressure, the Ministry of Education, Science, Sports and



Kato: universities should be privatized.

Culture (Monbusho), which had opposed the government's reforms, has announced that it intends to approve the plan, provided that the universities are managed under a different framework from other research institutes.

Monbusho officials say the pressure to reduce the number of civil servants was now too strong for the ministry to keep resisting the plan, as national universities have more than 125,000 staff, a sizeable proportion of Japan's civil servants.

But Monbusho argues that the current agency bill, which sets targets based on cost performance, could affect the standard of research and education.

The ministry plans to ask that the bill be modified or that there should be a separate bill for universities, so their priorities would not be dictated by financial performance.

It insists that the evaluation of each agency be carried out by an independent assessment body, which Monbusho plans to set up next year (see *Nature* 400, 704; 1999). It wants to see performance-related targets selected by the education minister.

Monbusho's committee on university reform (see *Nature* 400, 703; 1999) will meet today (16 September) to finalize its proposals. Monbusho is expected to announce its final decision at a general meeting of the deans of the national universities on 20 September.

This schedule has been criticized by many as being too strongly influenced by political factors. Hirokuni Ono, head of Tokyo University's staff union, says Monbusho "shouldn't be rushing its decision because of the LDP election."

"Japan spent a long time deciding whether to privatize its railway and telecommunications sector," says Minoru Oda, former director of the Institute of Space and Astronautical Sciences and a member of the Monbusho committee. "I don't see how the government could decide the fate of the universities in such a short space of time."

Asako Saegusa

Cuts force telescope closures at Kitt Peak

Washington

The Kitt Peak National Observatory in Arizona plans to shut down two of its five remaining night-time telescopes in a cost-cutting move that managers say is essential but which critics say will not solve the observatory's fundamental problems.

Operation of the two small telescopes — a 0.9-metre instrument and a Coudé feed telescope used primarily for high-resolution spectroscopy of bright objects — will be phased out over the course of a year.

Scrapping the two telescopes will save about \$300,000 a year in operations costs, says Sidney Wolff, director of the National Optical Astronomy Observatories (NOAO) in Tucson, which includes Kitt Peak as well as the Cerro Tololo Inter-American Observatory in Chile and the National Solar Observatory, also based on Kitt Peak.

More importantly, she says, the closures will allow observatory staff to focus more effectively on the mountain's three remaining telescopes: the 4-metre-class Mayall and WIYN instruments and a 2.1-metre telescope.

Although the Coudé feed telescope is lightly used, the 0.9-metre telescope is oversubscribed by a factor of five, and has been particularly popular when used with NOAO's MOSAIC wide-field CCD array.

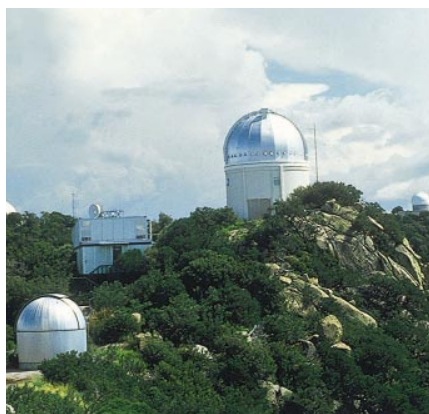
The closure of the 0.9-metre telescope is "a great shame", says John Bally, a University of Colorado astronomer who serves on the Kitt Peak users committee. The MOSAIC array can be moved to another telescope, but its availability will inevitably drop.

With decreasing budget allocations from the US National Science Foundation (NSF), NOAO managers have warned for several years of the need to shut down working facilities (see *Nature* 379, 569; 1996). A few smaller telescopes on Kitt Peak have already been retired. But opinions are divided as to whether this latest move is warranted.

Michael A'Hearn, a University of Maryland astronomer who headed a recent review of NOAO, says his committee "pretty much endorsed" the closure of the two smaller telescopes, although individual panel members opposed it. A'Hearn says the question of whether enough money is saved to make the closure worth it is a "debatable point" best left to NOAO managers to decide.

Wolff says she intends to "draw a line in the sand" after shutting down the two telescopes and would oppose closing Kitt Peak's three remaining night-time telescopes. But she wrote in the NOAO newsletter earlier this summer that the closure amounts to little more than a "temporary bandaid". There is broad agreement that the organization needs to change or face extinction.

With an annual budget of \$27 million, the NOAO consumes about a quarter of NSF's



Scope for savings: closing two of Kitt Peak's five night-time telescopes will save \$300,000 a year.

yearly spending for astronomy. Its traditional role has been to provide observing time for all US astronomers, particularly the estimated 50 per cent who do not have access to private telescopes such as those owned by the University of California.

As well as running Kitt Peak and Cerro Tololo, the NOAO is building a solar telescope for the National Solar Observatory and will handle community-wide proposals for the Gemini and other telescopes. But critics charge that the NOAO has not given the community any new, cutting-edge telescopes.

Bally thinks that the NOAO Tucson office is top-heavy with tenured scientists, and he would support a staff restructuring to rely more on interns and postdocs. The total NOAO staff for night-time programmes has

dropped from 200 in 1984 to 125 today, with many of the cuts in lower-paid positions.

A'Hearn says that Wolff is trying to make changes. A long-range plan, for example, includes several visionary projects, including the construction of very large (aperture of 30 to 100 metres) telescopes, large survey instruments such as the proposed Dark Matter Telescope, and a 'national virtual observatory' — a vast database of astronomical observations that would be made available to the whole community.

The committee led by A'Hearn endorsed this plan. Wolff says her staff are mostly behind the new thrusts, but "it's not 100 per cent, because it is a major change in direction".

The NOAO is facing an identity crisis, with some critics even questioning the continuing need for a national observatory. Wolff concedes that "there has never been a clear agreement on the mission of the NOAO", but believes there is more need than ever now for a national observatory.

Bally agrees that the NOAO is still needed, but would like to see it return to its "original service function" — building new instruments and providing telescope time to the wider community — rather than building up its own scientific capability.

The NSF, meanwhile, has asked Wolff and her staff to talk to managers of space-based observatories, such as the recently launched Chandra X-ray telescope and the upcoming Space Infrared Telescope Facility, to plan possible coordinated observations with NOAO's 4-metre telescopes. **Tony Reichhardt**

Iranian visit fosters links with US

San Diego

A group of Iranian scientific leaders last week visited the US National Academy of Sciences in Washington DC in the first scientific exchange since the two nations broke diplomatic relations 20 years ago.

After the visit, academy officials said they plan to send a delegation of leading US scientists to Iran next spring.

"Following many years without significant contact with our scientific counterparts in Iran, we heartily welcome this opportunity to explore future areas of cooperation," said Bruce Alberts, president of the US academy.

Academy leaders held a reception for the Iranian delegation, which included the president of Iran's Academy of Sciences, Reza Davari Ardakani, a philosophy professor from Tehran University, and the academy's vice-president, Mehdi Nejad Bahadori, a mechanical engineering

professor at the Sharif University of Technology.

The visit was arranged by the Washington-based Federation of American Scientists, which fosters international scientific cooperation, with the assistance of Ali Mansoori, a professor at the University of Chicago. Jeremy Stone, president of the federation, led a group to Iran last December as a prelude to the visit.

"Scientific exchange is a natural precursor to improved relations between nations without diplomatic relations," says Stone, who took part in similar scientific visits to China in 1972 and Vietnam in 1989.

Although there were no formal discussions at the meeting, officials say the talks laid the groundwork for future exchanges. Areas of discussion reportedly included renewable energy, education, earthquake hazards, the environment and public-health concerns. **Rex Dalton**

Russian scientist 'tried to smuggle spy device to China'

Moscow

A Russian scientist, Vladimir Shchurov, has been accused of attempting to deliver to China an underwater acoustic device which the security service claims could be used to track Russian submarines.

The accusation was made after Shchurov was detained at customs. Russia's Federal Security Service last week searched the laboratory Shchurov heads at the Pacific Oceanological Institute of the Far Eastern branch of the Russian Academy of Sciences, in Vladivostok. It was the security service's second visit to the institute within three months. The papers of a prominent ecologist there, Vladimir Soyfer, were searched in July (*Nature* 400, 300; 1999).

Shchurov's alleged action could lead to several years in gaol. But he denies any impropriety, and points with pride to the fact that his acoustic device, constructed 20 years ago and designed as a research tool, is still considered by the security service to be a threat to national security.

"Our acoustics laboratory is the only one in Russia that uses the unique technology developed here. But our scientific work cannot find any potential users in Russia," he says. "The contact with the Chinese has allowed us to prolong the research, to buy equipment, and to hire young and talented scientists. As a result, we have been able to make substantial progress with our research. But now attempts are being made to drive us out of the scientific market."

Shchurov argues that he did not need an export licence for the device because it was only to be taken out of Russia for the time needed to conduct experiments on two Chinese scientific ships in the Yellow Sea in October and November. The Russian Academy of Sciences had given permission for the work, he says.

Three years ago, Viktor Akulinychev, director of the Vladivostok institute, signed a contract with China's Harbin Engineering University in Heilongjiang province for testing the Russian techniques of studying underwater noises. Shchurov points out that no-one, including the security service, objected to the arrangement at the time.

"The use of such devices to investigate underwater noises has long been routine," says Shchurov. "The only difference is that this time we were to use Chinese, rather than Russian, scientific ships. Our own scientific fleet cannot afford even a short expedition." **Carl Levitin**

Four front runners in race to become director of Unesco

Sydney

The race to succeed Federico Mayor as director-general of the United Nations Educational, Scientific and Cultural Organization (Unesco) entered its final phase last week with the closure of nominations. Interviews by the 58-member executive board begin in Paris on 13 October.

Of the 11 candidates for the six-year appointment, all but one declared their bids several weeks ago. The exception is Daniel Janicot of France, who was nominated by Georgia last week. Janicot, at 51, is the youngest candidate and the only internal one, being an assistant director-general in charge of the directorate (known as Mayor's cabinet).

Two candidates, Koichiro Matsuura of Japan (61) and Ismail Serageldin of Egypt (55) began campaigning early and, having secured a number of national endorsements, are thought likely to poll well. A career diplomat, Matsuura is Japan's ambassador to France and knows Unesco well as chair of its World Heritage Committee.

Serageldin, vice-president of the World Bank, was nominated not by his native country but by Burkina Faso, reflecting his involvement in developing countries. As chairman of the Consultative Group on International Agricultural Research, he spoke at the Unesco/ICSU World Conference on Science in Budapest last June, where Sweden surprised many by declaring its vote for him from the platform.

The Saudi Arabian nominee, Ghazi Al-Gosaibi (59), is ambassador to the United Kingdom and a former academic. He claims to have up to 26 votes from Arab, African and non-Arab Islamic states.

The Australian candidate, Gareth Evans (55), is a lawyer and former foreign minister. He is close to completing visits to 55 members



Evans: pledges to cut Unesco bureaucracy.

of the executive board and the United States, which he believes can be enticed to rejoin Unesco, and promises to simplify administration and to select and target programmes more rigorously.

The sole female candidate, Rosario Manalo of the Philippines (63), stood unsuccessfully in 1987, when Mayor began his 12-year term. An experienced diplomat and currently secretary general of the Unesco national commission in her country, she will trade on votes from the Asia-Pacific region.

Pal Pataki, Hungary's ambassador to Unesco, and chairman of its executive board, gained exposure to Unesco delegates through his leading role in the World Conference on Science and is the only candidate with strong scientific credentials.

The Sri Lankan, Senake Bandaranayake (61), the Indonesian, Makaminan Makagiansar (70), Lawrence Deighton Carrington of Trinidad and Tobago (58), and Ion Caramitru of Romania (57) are all professors (of archaeology, social sciences, linguistics and drama, respectively). But none is expected to go past the early rounds of voting.

The Matsuura, Serageldin and Evans camps believe their candidates will reach the final rounds. Al-Gosaibi's claims might bring him closest to an early majority. After a maximum of five rounds of voting from 18 to 22 October, one name will emerge for recommendation to 185 member nations invited to the general conference, which concludes on 17 November.

Peter Pockley

Kansas evolution ban under attack

Washington

Two professional biology organizations have added their voices to criticisms of the decision by the Kansas Board of Education to eliminate the teaching of evolution from the school science curriculum (see *Nature* 400, 701; 1999).

The board of directors of the American Institute of Biological Sciences has issued a statement describing the decision as "unfortunate", and saying that it "serves only to prevent students from acquiring a comprehensive understanding of modern biology".

A separate statement from the officers

and council of the Society for the Study of Evolution describes it as a "retrograde step" to single out certain aspects of science as being unsuitable for the classroom.

"The processes of observation and inference used to support our current understanding of the age of the Universe and of biological evolution are the same as those used in all branches of science," says the statement. "If we abandon the usual procedures of acceptance or rejection of scientific hypotheses in one area of science, then the whole of science is in jeopardy." ■

German biotech lab in bid to silence critic

Munich

A long-running dispute over freedom of speech at the Institute of Molecular Biotechnology (IMB) in Jena, eastern Germany, has flared up again following a move to stop the former deputy director from publicly criticizing the institute's current research.

Günter Löber published an article in the May issue of *Laborjournal*, a science news magazine, criticizing what he claimed was the low level of the institute's scientific output. Löber, an east German scientist, helped to develop the IMB's scientific mission when it was recreated in 1992 from an institute of the former East German Academy of Sciences.

The article also argued that the institute's scientific and administrative directors had blocked attempts to develop cutting-edge research in the so-called field of 'evolutionary biotechnology' which had been central to the institute's original mission.

To avoid being taken to court for breaking his obligation to maintain silence about institute affairs after retirement, Löber has now signed an agreement promising not to make further public criticisms of the IMB, against a penalty of DM50,000 (US\$26,450).

But the institute's move has raised concern among scientists, who feel that freedom of speech is being curtailed. "We should be able to say what we feel is true," says the Nobel prizewinning chemist Manfred Eigen, who headed the IMB's founding committee and its international supervisory board. "It is an insult to science that a research institute, backed by a [state] research ministry, should force retired scientists not to speak [out]," he says.

Eigen himself resigned from the supervisory board in 1997, complaining that his own freedom of speech had been curtailed (see *Nature* 385, 761; 1997). During a leadership dispute at the institute, Eigen was told by the research ministry in the state of Thüringen only to speak to institute directors in the presence of a ministry representative. The other board members resigned in sympathy.

Much of the current controversy centres on the institute's research strategy. Previous disputes, which outsiders see as having been fanned by personality clashes, have resulted in the departure of all senior scientists hired to work in molecular evolutionary biotechnology, the identification of drug targets by following changes in gene expression resulting from evolutionary pressures.

Evolutionary biotechnology will conti-

nue, says Rolf Hilgenfeld, the institute's current scientific director, but at a lower level than originally envisaged. A new position of department head was advertised this summer.

He believes the 'evolutionary biotechnology' approach to drug design to be risky, however, preferring to give greater weight to the more "careful approach" of examining single specified genes as potential drug targets.

But Eigen and Löber claim that the institute's research is becoming mundane, contrary to political aspirations to make research institutes founded in east Germany after the country's reunification break the conservative mould of west German science. "Nothing is innovative now," comments Eigen.

Hilgenfeld denies allegations of scientific mediocrity, claiming that the atmosphere at the institute, and the quality of the scientific output, have "dramatically improved" since 1997. Hilgenfeld says he appreciates the "great contribution" of the first generation of scientists at the IMB — including Löber. But he is "sad that some of them are trying to run the IMB down as soon as they retire".

He says it was necessary to take a legal move against Löber to protect the IMB's reputation. He was worried that Löber's "campaign" had been launched in a bid to influence the institute's evaluation next month by



Germany's science council, the Wissenschaftsrat. He also points out that Löber immediately agreed to the injunction and willingly agreed to pay half the legal costs.

Löber says he was not planning a prolonged campaign, which was one reason why he was prepared to agree not to make further criticisms in public. But Peter Schuster, a former research director at the IMB who left during one of the disputes, sees the step to silence Löber as more harmful to the institute than Löber's public comments. "It is scandalous, and bad for the image of science," he says.

Quirin Schiermeier & Allison Abbott



Eigen: 'don't try to gag retired scientists'.

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Evolutionary biotechnology will conti-

Laser project 'faces optics hurdle'

Washington

The Lawrence Livermore National Laboratory in California has changed the top three managers of the National Ignition Facility (NIF), a \$1.2 billion laser project under construction there, and is reviewing the project to determine the full extent of its difficulties.

The US Department of Energy (DoE), which owns the lab, has said that one issue — the need for clean-room conditions in the laser assembly area — accounts for most of the cost overruns on the project, which it admitted this month ran to hundreds of millions of dollars (see *Nature* 401, 101; 1999).

But some observers say that the project is dogged with other, harder technical problems, including difficulties in manufacturing several thousand large, precision optical components for its 192 laser beams.

Last week, Livermore officials were unavailable to discuss the optics or the clean-room issues. But they are expected to do so after an initial project review by the new management team.

The clean-room issue came to light after Ed Moses, then head of another laser project at the laboratory, began an internal study of the laser assembly plans in January. By April

he had concluded that the existing plan would not provide a clean enough environment.

Since then, project officials have been arguing about what to do, with Moses consulting outside experts, including engineers from Intel, the microchip manufacturer.

As this argument raged in June, both Bruce Tarter, the laboratory's director, and Bill Richardson, the energy secretary, were stating in public that the project was on time and within budget. The argument only became public after Mike Campbell, the laboratory's associate director for lasers, resigned on 27 August, ostensibly because he had not told the lab that he never completed his PhD.

As Campbell quit, Tri Valley Care, a laboratory watchdog group, told reporters and senior government officials in Washington that the NIF was up to \$300 million over budget. Richardson confirmed this within days, blaming the lab, and announced that an outside contractor would be brought in to ensure clean-room conditions during NIF assembly. Two other top NIF managers were replaced, and Moses took over as NIF project manager.

But Tri Valley Care claims that Campbell's resignation and the clean-room issue are a smokescreen that masks deeper



Time to come clean: critics say that the NIF's troubles go beyond clean-room difficulties.

► problems. The watchdog group says its information comes from Livermore scientists worried about how the problems with the NIF are eating into other research budgets.

"The cleanliness problem is by no means the only problem," says Marylia Kelley, president of Tri Valley Care. "Some lab staff are derisive about that being the only problem."

Kelley says the optics — especially the potassium dihydrogen phosphate crystals used to generate the lasers — cannot be produced to sufficient quality and have been failing under test. The NIF specification required rapid-growth crystallization, ten times faster than that used in previous laser projects, to produce these components at the right cost.

One senior NIF scientist, speaking in Washington last week, was optimistic that the optics will be working in time. But the Livermore lab refuses to discuss the issue, and the DoE's denials of these problems are less than complete. "All of my information is that it is the complexity of the laser infrastructure, and not the optics" that have caused the cost overruns, says an official who has been speaking for Richardson on the issue.

The House of Representatives Science Committee will probably ask the General Accounting Office to investigate the NIF. The new management team and a panel of outside experts to be assembled by Richardson will determine where the project goes next.

The DoE says that the problems will have no impact on planned NIF collaborations in weapons research with the United Kingdom and France, except insofar as they delay the project's completion.

But non-weapons scientists fear that the overruns will move the NIF towards weapons research at the expense of experiments into obtaining energy from inertial confinement fusion. "It has gradually been happening anyway," says Steve Dean of Fusion Power Associates, a fusion-energy lobby group.

Observers say that the new managers are primarily weapons people. Also, a reduced-specification NIF that fails to achieve ignition — a prospect which Livermore officials privately acknowledge — will be more useful to weapons scientists than to fusion-energy researchers, who need ignition to prove the potential worth of inertial confinement fusion as an energy source.

Colin Macilwain

France set to cut search for small extrasolar planets

Paris

Only a few years after the discovery of giant, extrasolar planets, a proposed French mission to extend the search to smaller planets in 'habitable' orbits is at risk of being axed. The project (see *Nature* 400, 316–317; 1999) looks set to fall foul of cuts imposed on the national space agency CNES by the science ministry.

The main scientific goal of the proposed Corot space telescope, which was due for launch in 2002, is to use seismology to study the internal structure of stars other than the Sun. But, with the telescope designed to fix the same part of the sky for 150 days at a time, astronomers built into the mission a second goal — detecting extrasolar eclipses.

Observing such eclipses would yield the orbital period and size of any planets with precision, says Frederic Bonneau, head of the mission at CNES.

But the mission, costed at a modest FF350 million (US\$55 million), including launch costs, is considered the primary target for cuts worth FF200 million to be imposed on CNES next year. The agency's science programme committee plans an emergency meeting next week to discuss Corot's future.

One member of the panel says it is likely to confirm its support for the mission, with pressure to axe Corot coming from the science

ministry, which is keen to trim France's commitments to big science programmes, and national projects in particular.

The panel member adds that space agency officials are in discussions with the ministry to try to win a reprieve for the mission. Other options to be considered at next week's meeting of the committee include postponing Corot, trying to reduce its costs, and searching for partners.

David Hughes, an astrophysicist at the University of Sheffield in the United Kingdom, is sceptical of Corot's chances of detecting planets, arguing that extrasolar eclipses are rare, even when one is looking at 6,000 stars. By definition, an eclipse would also be irreproducible, he points out. But he agrees it is a worthwhile secondary scientific objective. "If there is a reduction in brightness of the star, the data will hit them in the face anyway."

But Hughes describes Corot's main scientific goal as "super science". He argues that solar seismology has revolutionized stellar science by allowing researchers to "probe in depth" into the interior of stars, yielding information on pressure and temperature.

Because Corot would take the technique to other stars for the first time, "it is one of these space missions that has to happen," says Hughes. "If the French don't do it first, someone else will."

Declan Butler

Call for UK biotechnology centre

Sheffield

Britain's biotechnology industry last week called for the creation of a National Biotechnology Centre to boost confidence in its activities. In a report based on the findings of a government-funded delegation to the United States, the BioIndustry Association asked the government to create a centre to encourage and coordinate investment in biotechnology.

The report says that the US biotechnology industry has been encouraged by a supportive regulatory and planning environment, a responsive academic community and financial incentives.

The suggestion has already been backed by Sir Richard Sykes, chairman of Glaxo Wellcome and president of the British Association for the Advancement of Science. Speaking at the opening of the association's annual conference in Sheffield on Monday (13 September), Sykes said that such a centre was needed "if we are not to fall further behind the USA or be caught by Germany".



Sykes: looking for government backing.

Sykes accused the government of inconsistency between its messages and its actions with respect to the biotechnology industry, citing its recent refusal to approve the Wellcome Trust's plan to expand its genome campus near Cambridge (see *Nature* 400, 803; 1999).

"The Wellcome Trust is consequently looking elsewhere, including overseas, to find alternative suitable sites," he said. "A chance to create the infrastructure for exploiting our leading-edge science may have been missed."

Sykes added that Britain had fallen behind in the struggle to compete with developed and some developing countries. He said it was up to the government to create the right climate for Britain to prosper through science.

Natasha Loder

Moves are afoot to probe the lake trapped beneath Antarctic ice

Lake Vostok, isolated from the rest of the biosphere for at least a million years, could be the subject of an international initiative to plumb its secrets.

Some 80 scientists from 14 countries will meet in Cambridge, England, next week to formulate plans for exploring one of the world's last uncharted natural wonders: Lake Vostok, which is buried beneath four kilometres of ice in East Antarctica.

Interest in the lake stems mainly from the fact that it has been isolated from the rest of the biosphere for at least one million years — some researchers say perhaps 35 to 40 million years. "The driving force behind this effort is the prospect of finding organisms that have evolved in ways we might not have considered possible," notes Todd Sowers, a palaeoclimatologist at Pennsylvania State University.

Indeed, the US space agency NASA is interested in the exploration of Lake Vostok for a related reason: it offers a 'testbed' for the search for alien life in the ice-covered ocean thought to exist on Jupiter's moon Europa.

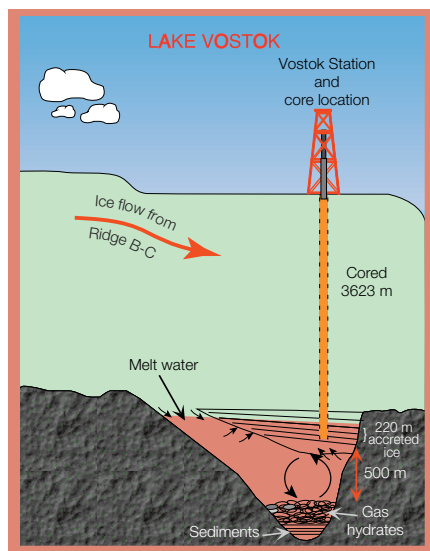
The full extent of Lake Vostok was not appreciated until 1996, when analysis of radar and seismic data revealed that it is 200 kilometres long, covers some 14,000 square kilometres and is up to 500 metres deep (see *Nature* **381**, 684–686; 1996).

Last year, following discussions with the Scientific Committee on Antarctic Research (SCAR), scientists completed work on the Vostok ice core, stopping at a depth of 3,623 metres — about 120 metres above the ice/lake interface — to avoid contaminating the lake's water with drilling fluid.

The lake is still untouched, but scientists have been working on a safe means of probing its hidden reaches. The Cambridge meeting, to be held at Lucy Cavendish College, is sponsored by SCAR. It "intends to get hard answers to crucial scientific questions about the lake and to establish a format for an international research programme there", says conference chair Cynan Ellis-Evans, a microbiologist with the British Antarctic Survey.

'Curiosity' or scientific target?

The meeting follows a workshop in Washington last November, sponsored by the National Science Foundation (NSF), which addressed the question of whether Lake Vostok is merely a 'curiosity' or an important target for scientific investigation. "A consensus emerged that there were compelling scientific reasons for studying this lake that go well beyond the thrill of exploration," says Robin



Going down: so far, exploration of the ice above Lake Vostok has stopped 120 m above the ice/lake interface, owing to fears of contamination.

Bell, a geophysicist at Columbia University's Lamont-Doherty Earth Observatory, who co-chaired the NSF workshop.

One goal of the Cambridge meeting, says Bell, is to formalize the scientific rationale for exploring Lake Vostok and to draw up a more rigorous agenda for studying the lake. "We've got to bring everybody up to speed and to identify the main gaps that need to be filled before we can take this to the next level and begin actual exploration," adds Sowers.

The general outlines of a plan are already emerging. The first task will be a geophysical site survey — to be conducted by Bell and colleagues — that would use new radar, seismic, gravity and magnetic data to map the boundaries of the lake and help discover its origins.

The depth of the lake and sediment thickness would be measured and salient features such as hot springs identified. The discovery of hot springs would be an important breakthrough, says Michigan State University microbiologist James Tiedje, as it would reveal a source of energy that could sustain life in the lake.

The next step would be to lower observing instruments into the lake. This would be done by using a standard hot-water drill to carve a hole down into the ice to within about 100 metres of the lake surface.

A bullet-shaped probe — carrying a suite

of instruments, cameras and perhaps small tethered robotic submarines — would use a heated tip to melt its way through the last stretch of ice, sealing itself in from behind upon freezing. Information would be relayed by cable to the surface.

The final phase would be to use deep coring techniques to drill into the lake in order to retrieve water and sediment samples for examination in the laboratory. "Many aspects of this programme have never been done and have no documented approaches," a NSF panel concluded last year.

Keeping clean

Accessing the lake in a non-contaminating fashion will be a challenge, says Bell, "as you have to worry about getting both in and out cleanly. We also have to find a way of keeping a hole through the ice open without freezing in — something that has never been done before in such thick ice sheets."

All told, says Ellis-Evans, "this will be an enormous undertaking, on a scale unprecedented in Antarctica". Perhaps two dozen cargo flights would be needed merely to bring in the equipment necessary for hot-water drilling, he says, and even more to deploy the larger drill for obtaining samples.

That leads to the other critical goals of the Cambridge meeting: finding ways to finance such a large-scale effort and establishing an organizational framework for doing so. "SCAR is stepping forward by running this workshop," says Bell, and may therefore be the logical choice for the coordinating role.

On the funding front, NSF has agreed to pay for the geophysical site survey. "Everything hinges on getting that survey done," says Bell. He says that once it is under way, which could be within a year or two, coring could begin within about five years.

But NSF is not prepared to fund the entire exploration, says glaciologist Julie Palais, the foundation's Antarctic programme manager. "This is a big international effort and we're just one part of it." Palais is attending the Cambridge meeting to gauge the scientific interest from other countries and to see how much money they are willing to contribute.

"It will be a real challenge to pull this off, so we need to make a strong scientific case," Sowers says. "The money won't come unless we can convince people that this is a worthwhile endeavour."

Steve Nadis

ROBIN BELL

Los Alamos officials disciplined over weapons spy scandal

Washington The University of California has announced mild sanctions for three officials at the Los Alamos National Laboratory in New Mexico who were caught up in the scandal over the alleged leaking of nuclear secrets to China. The trio were accused by the Department of Energy of failing to respond expeditiously to still-unproven allegations that Wen Ho Lee, a scientist at the laboratory, had been responsible for the leaks.

The university said that one of the officials — believed to be Sig Hecker, the former director of the laboratory — will have a letter put in his file from its president “regarding his responsibilities at Los Alamos during the security investigation”. Two counter-intelligence officers were also sanctioned, with one of them having his salary frozen and “job assignment restricted for five years”.

NASA's orbiter on target to reach Mars next week

Washington The latest in the US space agency NASA's line of spacecraft to explore Mars is scheduled to arrive in orbit around the planet next Thursday (23 September). The Mars Cli-

mate Orbiter, launched last December, will spend two years monitoring the martian weather with a wide-field camera and a radiometer to measure atmospheric temperature, dust, water vapour and cloud cover. The orbiter will also act as a radio relay for the Mars Polar Lander, which is set to touch down near the martian south pole on 3 December.

Drosophila genome sequencing finished

Washington US gene sequencing company Celera announced last week that it has completed the sequencing phase of its collaborative programme with the Berkeley *Drosophila* Genome Project to identify the genome of the fruitfly.

Celera, based in Rockville, Maryland, said that its banks of PE Biosystems machines will now move on to start sequencing the human genome. The machines have sequenced gene fragments amounting to 1.8 billion base pairs as part of the *Drosophila* project. Celera and the University of California at Berkeley will now use computer algorithms to assemble ‘shotgun’ fragments into a complete sequence for *Drosophila*.

“While sequence assembly is a challenging process, we are optimistic about our ability to complete this phase in the near future,” says Craig Venter, Celera's president. Celera

says it will start making sequence data available next month, and that the full sequence will be published in “early 2000”.

Japan's space agency suffers another setback

Tokyo Japan's National Space Development Agency (NASDA) last week postponed the launch of its H-II rocket for the third time in a month. The agency said the launcher carrying the Multi-functional Transport Satellite had problems with the sensor for its fuel tanks.

NASDA had hoped to improve its reputation through the project, following several failures last year, including the launch of the world's largest telecommunications satellites, which failed to reach geostationary orbit (*Nature* 392, 321; 1998). NASDA now plans to launch the rocket after 17 September.

End in sight for polio in Asia-Pacific region

Tokyo Asian-Pacific countries, including China, Japan, Vietnam and Australia, are planning to announce the total eradication of poliomyelitis by autumn 2000, according to the Western Pacific regional committee of the World Health Organization (WHO). The move is expected to be confirmed at WHO's five-day regional committee meeting in

Macau, which opened on Monday (13 September).

In 1988, WHO launched a global initiative to wipe out polio by the year 2000. Global polio incidence has dropped by more than 90 per cent since then; the last known cases in the Asian-Pacific region were in Cambodia and Vietnam, in 1997.

Stalemate in talks aimed at worldwide ban on DDT

Paris Talks between 115 nations have made some headway under a United Nations treaty aiming to ban 12 of the most dangerous chemicals and pesticides, but have not reached a conclusion over DDT. The main controversy during negotiations last week in Geneva revolved around the pesticide. Malaria experts claim that, without effective alternative methods to control the disease, DDT should remain in use.

The Malaria Foundation International issued a statement signed by hundreds of scientists and a handful of Nobel laureates saying that "setting a firm deadline to ban DDT places an unethical burden on the poorest countries". In addition to DDT, representatives are still thrashing out the future of PCBs, dioxins and furans. Negotiations will resume next spring in Bonn, Germany, and could end late next year.

Wellcome invests in functional genomics

London Britain's Wellcome Trust announced last week that it is to spend £100 million (US\$167 million) over the next five years — roughly five per cent of its total expenditure — on a programme of 'functional genomics'. The programme will build on the results of the Human Genome Project, of which the trust is one of the principal sponsors.

The programme will also cover emerging technologies for high-throughput screening, and large-scale collections of biological materials and bioinformatics. "We believe the programme will build a crucial bridge between scientific studies and the remedies required to prevent and cure serious illness," said trust director Mike Dexter.

Polar bear study raises alarm over Arctic pollution

Munich Environmental scientists in Denmark are warning that the discovery of the first hermaphrodite polar bear in Greenland could mean that the stock of around 100,000 polar bears in the Arctic is seriously threatened by environmental pollution. The phenomenon is believed to be the result of the ingestion of organic poisons such as polychlorinated biphenyls (PCBs) through the food chain.

The levels of PCBs in the blood of seven hermaphrodite polar bears found since 1990 on the Norwegian island of Spitsbergen were six times higher than those found in bears in the Canadian Arctic. The Danish Institute of Environmental Research recently started a research programme to investigate the health of the 5,000–10,000 polar bears in Greenland. One of the four bear cadavers investigated had both male and female sexual organs. "If this means that 25 per cent of polar bears in Greenland have deformed sexual organs, it would be alarming," says Christian Sonne-Hansen, head of the research project.

Freed meteorologists come in from the cold

Moscow Two meteorologists captured by Islamic extremists who crossed into Kyrgyzstan from Tajikistan are reported to have been freed after their abductors learnt that they were scientists — and concluded that no one was likely to pay for their release.

The researchers, part of an expedition studying the Abramov glacier in the Pamir Mountains, were freed after their money, documents and warm clothes had been taken. After descending to safety, one said: "We have now become accustomed to surviving not only without salaries, but also without clothes."

Biotech companies must get back to basics to weigh up risks

Sir—A substantial acreage of US fields is now given over to transgenic plants that produce an insecticidal protein (a derivative of the *Bacillus thuringiensis* insect-control protein CryIA, also known as *Bt* toxin). In an effort to restrict the spread of resistance to *Bt* toxin, the use of refuges—areas of non-transgenic plants planted close to the transgenic variety—has been adopted. In *News and Views*¹, M. J. Crawley wrote: “The strategy might be expected to work because resistance is usually a genetically recessive trait”.

However, F. Huang *et al.*² have recently shown that, for the European corn borer, resistance is an incompletely dominant trait. Although the significance of these results has been challenged³, they may mean that refuges will not work for this insect, a major agricultural pest in the United States.

This is something that should have been established before the widespread planting of transgenic corn. It's hardly surprising that there is distrust of agri-biotech companies⁴, when they do not put significant effort into answering the basic questions that enable such risks to be evaluated.

Jonathan Ewbank

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California's libraries get wired up to e-journals

Sir—I would like to respond to the article by Rex Dalton about the acquisition of electronic journals at California State University (“Bumpy ride for ‘core e-journals’ project,” *Nature* **400**, 200; 1999). It is true that CSU will license fewer e-journals for its Journal Access Core Collection (JACC) than were initially projected. But the future of the project is by no means uncertain. Indeed, we consider our efforts highly successful. This project has laid the groundwork for other universities to follow in the future. For CSU it represents the first of what we hope will be many attempts at licensing additional e-journal resources which conform to JACC requirements.

Dalton's statement that the JACC

project is “widely seen as a radical move with little faculty input” is inaccurate. The selection of 1,300 titles for JACC was based on the fact that 15 or more of the 21 CSU libraries were already subscribing to those titles in print. Faculty members have always had input to the selection of journals at CSU libraries and they all look forward to the many benefits that our transition to the electronic form will bring.

Finally, we understand that some libraries remain wary about dropping print subscriptions and are taking a wait-and-see approach to JACC. But this attitude is universal and it's not just about JACC. It's about the entire medium and has everything to do with the fact that many publishers have yet to consider the realities of a changing market and the needs of their customers.

Evan A. Reader

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Sensible precautions make good science...

Sir—Søren Holm and John Harris strongly criticize the precautionary principle but they seem not to understand it (*Nature* **400**, 398; 1999). They complain that it is not valid for evaluating evidence, when that is not what it is for. It is a tool for decision-making, and, like many such tools, deals in expectations rather than probabilities.

The point is that it requires us to take into account not just the probability that a technology will be hazardous, but also the benefits if it succeeds and the costs if things go wrong. There may have been a very small probability that a large ship travelling at high speed in the North Atlantic would hit an iceberg, but the captain of the *Titanic* should have thought more about what could happen if it did—and all the more so because it didn't really matter if the voyage lasted a few hours more.

Holm and Harris argue that the precautionary principle would have stopped us developing genetically modified organisms (GMOs) because the greatest uncertainty about their possible harmfulness existed before anybody had produced one. But the principle does not demand that we halt research if we cannot be certain the end result will be safe (though common sense suggests it is unwise to make large investments if the end result is likely to be dangerous). It is to be applied at each stage in the process, weighing the risks in going one step further against the likely benefits if the project is successful.

That is why we and many others are arguing not for a complete ban on research into GMOs but for a five-year moratorium

on field trials and commercial planting. There is a lot more research to be carried out in the relative safety of a closed laboratory first. This is always good practice, but it is especially important in the case of GMOs because of the irreversibility that is inherent in the technology. If a new drug proves to be harmful we can withdraw it, but once genes have left the laboratory there is no calling them back. The experiments in which GM milkweed was found to harm the monarch butterfly were performed in contained conditions; had this been discovered in field trials, the gene might already be spreading through the environment.

Our objection to the current field trials of GM crops is based not on whether commercial planting would be safe (though we are concerned about that), but on whether the trials themselves are safe—and whether they are well enough designed to be worth the risk. Neither has been shown to be the case. At the end of a moratorium, a much better-informed risk assessment should be possible.

C. Vyvyan Howard*, **Peter T. Saunders†**

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...and can mean saying 'yes' to innovation

Sir—As conveners and participants in the conference that issued the Wingspread Statement we would like to respond to the Correspondence by Holm and Harris. The precautionary principle does not instruct us to balance evidence in a specific way. It requires us to evaluate honestly all the evidence and the uncertainties. The precautionary principle comes into play when decision-makers suspect that a course of action may have harmful effects but are uncertain about their cause and possible extent. It demands more, not less, science in decision-making, relying on multiple lines of evidence from diverse disciplines and constituencies of interest.

Holm and Harris contend that the precautionary principle will block innovation. On the contrary, application of the principle may result in saying ‘yes’ as well as ‘no’. ‘No’ does not always mean a prohibition, but can mean any of an array of measures to prevent harm. For example, precautionary action may consist of restrictions on use and emissions pending further examination, a requirement to monitor for impacts of an activity as a condition of going ahead, cradle-to-grave product responsibility, labelling, or requiring a proponent to examine alternatives to the use of a potentially dangerous chemical or activity.

Methods exist for regulatory approval of

new technologies consistent with the principle. Assurance bonds, pre-market testing and post-market surveillance allow us to move forward carefully but to shift the responsibility for harm to the proponent of a technology. The gains achieved through 'clean production' methods provide evidence that implementation of the precautionary principle stimulates, not stymies, innovation. Clean production involves the prevention of harm at source through the use of less material-intensive and toxic production systems and products, and was a logical outcome of the principle's demand for preventive action in the face of uncertainty. The question asked is switched from 'how much pollution is acceptable?' to 'how much can we prevent?'.

Holm and Harris suggest that we wait for damage to occur before taking action. Unfortunately we already have a hole in the ozone layer, marine fish stocks are depleted, and climate change threatens future generations. The challenge is to prevent harm before it occurs.

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Sweden's answer to genomics ethics

Sir—DeCODE genetics, the Icelandic genomics company, objects¹ to critical viewpoints on its ethical practices in a News article². DeCODE also criticizes the favourable review of the ethical practices of UmanGenomics, a Swedish genomics company. We disagree with deCODE's distorted description of UmanGenomics^{3,4}.

DeCODE says that its ethical guidelines are better than those anywhere else. However, UmanGenomics has a unique formula for handling ethical issues, developed in parallel with the ethical guidelines for use of genetic biobanks published by the Swedish Medical Research Council. This procedure was correctly described in the News article.

UmanGenomics developed a unique ethics formula fully acceptable to the individuals in Västerbotten county because it is these people who made UmanGenomics' business possible. Another reason is that UmanGenomics' customers, pharmaceutical companies, are known to refrain from collaboration with organizations that may draw them into questionable ethical issues.

It is not clear why deCODE states that "government committees and bureaucrats"

granted UmanGenomics permission to use its medical bank. UmanGenomics does not own a biobank, as explained in the News article. The collaboration between the Medical Bank in Umeå and UmanGenomics is regulated by a business agreement, as is common practice between two legally separate units.

Without having access to the shareholders' agreement, and thus no knowledge about how the ownership of UmanGenomics may be exercised, deCODE gives the impression that the proper use of the biobank is not assured as "governments have a bad record on violation of privacy"! This statement is totally out of context.

Sune Rosell

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Ethics training more important than ever

Sir—Scientific enterprise is built on a foundation of trust, and research ethics are the cornerstones: they define the boundaries of responsible conduct and sustain further enquiry. Ethics are of increasing importance in today's competitive environment as the barriers between industrial and academic research diminish. Yet young British scientists often receive no formal training in research ethics.

Many students are exposed to ethics only through the example of their mentors as issues arise. I believe that ethical principles and the skills of ethical analysis should be taught explicitly to graduate students, and then reinforced by example.

I benefited immensely from the mandatory instruction in research ethics¹ that I received as a graduate student at Johns Hopkins University in Baltimore, Maryland. So, recently, I led a discussion on ethics for UK biology graduate students. The students were given copies of *On Being a Scientist: Responsible Conduct in Scientific Research*², which deals with issues such as conflicts of interest, subjectivity and bias, credit and authorship, and misconduct. The students enthusiastically discussed the principles of ethics, analysed the dilemmas, and shared personal experiences. The event was so well received that next year it will be expanded into a series of discussions. I hope that faculty members at other institutions will start similar programmes.

John T. Finn

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Latin America treats science as a curiosity

Sir—Latin American politicians rarely have a clear understanding of the role that science and technology play in the modern world: these are simply seen as parts of the political game.

Commitment to those in power counts for more than professional expertise when it comes to both research funding and appointment to decision-making positions. As a result, research activities are plagued by disruptive political instabilities. Funding is not only scarce, but poorly distributed and badly spent: programmes are established without clear scientific objectives and money is given to researchers who lack the right scientific background.

This pattern may not apply to some Latin American institutions, but it is generally valid and does much to explain why Latin America's contribution to the production of knowledge is so small.

National research councils have been established throughout the region, along with modern universities with research programmes. But efforts have been mainly directed towards maintaining this uppermost level of scientific activity. Science education has never been given priority in state schools. There are few science museums for the public or specialist journalists who can spread science news in an accessible way.

This situation reflects an official belief (never explicitly expressed) that Latin America needs only a limited number of top scientists, not a scientifically literate population. It permeates research agendas and budgets, encouraging advanced projects without thought for the limitations of local expertise and industrial infrastructure, leading to frustration and wasted resources. The authorities neglect to develop modest programmes that could help strengthen a scientific culture.

Without decisive action in this latter direction, science in Latin America will continue to be a curiosity and, at most, a source of personal prestige for some gifted scientists. The ever-widening technological gap that separates us from the industrialized world will not be filled without bridges between popular thought and the language of science.

One of the greatest challenges in establishing an independent research capacity in developing countries is to support both the spread of scientific culture and the strengthening of local research teams. Both deserve adequate funding and training.

Ivan Chamboleyron

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Towards a more venturesome Europe

Europe has been slow to exploit its considerable scientific research expertise for economic benefit. A culture of venture capitalism must be created if Europe is to compete more effectively with the United States.

Craig Pickering

Europe is often said to have inflexible product and labour markets and a poor record on innovation. Unfavourable comparisons are most frequently drawn with the United States. Between 1975 and 1995, the turnover of 24 US companies, mainly in high-technology markets, increased from \$2 billion to \$248 billion, creating more than 1.3 million jobs. Apart from pharmaceutical companies, where are the European equivalents?

The financing of early-stage high-technology companies is a prime case in point. Europe must do much better. Solutions have been proposed covering tax, universities, scientists, high-tech companies themselves and, above all, the big financial institutions and the equity markets in which they operate. What can Europe learn from the US experience? The most obvious answer is to improve access to capital and encourage scientists to become at least part-time entrepreneurs.

The gap

Venture capital in this market illustrates how far Europe has to go. In 1992, US funds serving the early-stage market were about seven times greater than in the United Kingdom (compared with a 6:1 difference in gross national product). By 1996, the gap had hardly shrunk. The United Kingdom and the rest of Europe need a far better understanding of the size and performance of high-technology industries. The UK government, for example, publishes headline statistics on the leather industry, but nothing on the high-technology sector overall.

There is clearly an economically significant gap between the United States and Europe. The Organization for Economic Cooperation and Development (OECD) produces a series of statistics giving countries' 'technological balance of payments' (providing a measure of trade in technologies), which deserves more attention than it gets. In 1994, the latest year for which figures are available¹, the United Kingdom had a surplus of £362 million (US\$581 million), Germany was over £1 billion in deficit and Italy £0.5 billion in deficit, whereas the United States was nearly £11 billion in surplus. Point made? Well, not quite, but this snapshot suggests that there is a lot of truth in the conventional wisdom that the United States is a thriving technological economy that draws on scientific expertise, whereas Europe is much less so.

Behind the statistics, industries on the two sides of the Atlantic differ radically in their approach to the projects they aspire to finance. Silicon Valley's venture capitalists — or private equity providers, as they now like to be known — are locked into the local and national economy in ways that Europeans can hardly dream of at present. The East Coast equivalents do much the same. A US scientist who aspires to be a high-tech entrepreneur can use a well-known and well-established infrastructure. The Red Herring Club is a good example. In this, every year would-be entrepreneurs and finance providers gather to allow one to pitch to the other. Such events are beginning in Europe, though on a tiny scale.

The entrepreneurs themselves also differ, in both numbers and culture. Although the facts are not well documented, it is clear that US scientists are much more likely than their European counterparts to start a business. Compare the origins of US biotechnology firms such as Genentech with the excellent contribution of Tim Berners-Lee of CERN, the European Laboratory for Particle Physics, to kick-starting the World-Wide Web.

Corporate venturing has reached a high status in the United States. It generally involves a large corporation taking stakes in a new venture that in some way promotes the large company's commercial agenda. Intel, for example, has a strategy for developing such relationships and terminating them amicably when its strategy dictates that it is time to part. European companies do undertake such deals, particularly in the

pharmaceutical industry; the difference is in the scale and sophistication.

Government policy is also relevant. The US government has provided a more benign tax regime than its European counterparts. This can be judged by indicators such as the use of capital-gains tax to encourage early-stage investments.

Why the gap matters

A case needs to be made for focusing on high-tech and early-stage investment in Europe. This may be obvious to those in the scientific and technology community, but it is not so to key players in financial institutions and government. Focusing does not mean feather-bedding or doing down other sectors, but helping high tech to realize its potential.

Key arguments include: (1) We don't have a global economy yet, but it is developing much more rapidly than could have been conceived of a generation ago. The economic crisis that began in Asia last autumn touched Russia and Latin America in a matter of days. The Internet is shortening communication lines and reducing distribution costs across national borders in ways whose impact we can only dimly discern. Technology is thus intervening in national economies on a rapidly increasing scale.

(2) New technologies create jobs. With unemployment a huge political, economic and social issue in Europe, this is a crucial point. The European Commission's seminal 1998 risk-capital paper² cited several surveys showing that US and European early-stage



Those who dared won: success stories such as Genentech's are far less common in Europe.

companies are more likely to create jobs than the average.

(3) In 1949, no one could have appreciated the impact of the PC, the Internet, bioindustry, new materials and all the rest over the following 50 years. The markets must be persuaded that early-stage investment in high technology will reap rewards. There are risks, of course, but good management of these investments should and can pay off. The UK venture-capital industry says' returns on UK venture-capital funds in 1998 out-performed those on UK pension funds and the top 100 companies on the UK Stock Exchange.

There are risks here for entrepreneurs, funding sources, scientists and employees. These can never be removed entirely. But a coherent policy in Europe towards encouraging early-stage, high-technology ventures could do much to smooth the path.

Where the money is

The funding of research and development (R&D) is a key benchmark. In 1996, according to the OECD, the US economy spent £117.7 billion on R&D, whereas the four largest European Union (EU) states together spent £60.9 billion. The bulk of this funding, in all five countries, came from the business sector: £85.5 billion from US companies and £40.7 billion from European companies. There is a role for both public and private sectors: among 400,000 US industrial patents, the key initial research was publicly funded for 73% of them, leaving a sizeable minority for the private sector.

Where did this private funding come from? In general, it came from companies' own resources and from giant financial institutions, rather than from individual savers and investors. Banks predominate in such investment in mainland Europe, and they are joined by US and UK pension funds (mainland Europe is increasingly taking the same route). UK insurance companies, for example, were managing £750 billion in 1998.

This money should be seen by scientists as a potential funding stream for ideas they have with commercial potential. It will not always fund the most basic research, although companies account for a growing proportion of research papers published in scientific journals. Such money may also be available much sooner than has traditionally been the case. Venture capitalists may act as intermediaries, and a new species of technology broker could soon emerge. They would understand the technology and science well enough to explain it to potential funding sources, and enough about commerce to explain to scientists, to be able to promote deals in science.

Policy levers

If it is a priority to change the culture among financial institutions and their advisers as well as in the science base, levers held by both communities can operate directly, and

governments can provide incentives. There are many sides to this issue, but two important aspects are improving access to capital from those sources and encouraging an entrepreneurial culture and thus a greater number of potential investments.

There are signs that access to capital in Europe is changing. But adequate information about early-stage ventures is a key issue. Actuaries, and to a lesser extent accountants, are grappling with the issue: evaluating early-stage investments is a hot topic among actuaries, who are debating how they can optimize potential income and capital-gains money from these companies. Accountants are looking at 'intangible' assets, trying to measure intellectual property more accurately on balance sheets. Universities throughout Europe are looking to California and the Boston area for models, lessons and inspiration.

But much more is needed. There is a growing view across Europe that there is a case for tax interventions where markets are not operating efficiently. The French government has introduced the DSK scheme, an imaginative attempt to give insurance companies and policyholders incentives to invest in early-stage ventures. The United Kingdom is building on two decades of experience in encouraging capital for new ventures. Persuading conservative financial institutions to be more open-minded about early-stage high-technology investments should be a priority. The Williams report¹ advocated a tax incentive to UK insurance companies to invest in this direction. The report's conclusions should be considered by all the European countries.

Europe needs to set up tax systems that enable early-stage high-tech ventures to obtain finance with the same ease as occurs in the United States. But the debate over tax harmonization across countries tends to hamper this. There is a case for a common EU regime that is easy for investors to understand, but this proposal always arouses strong feelings. Member states should at least share experience and best practice. With a new European Commission just starting, the timing is good for such initiatives.

Public money is also being used. The German government has put considerable funding in recent years into kick-starting its late-developing but fast-growing biotechnology industry. The UK government is collaborating with the Wellcome Trust and the Gatsby Foundation to fund University Challenge, which aims to encourage venture-capital skills on campus.

Like the tax system, public expenditure can address market failures and imperfections. It can also address wider policy objectives. (So can tax, but traditionally it has not been used much to pursue science policy.) Here it is sheer volume, rather than commitment as measured, say, by the proportion of

overall government expenditure on science, that keeps the United States ahead of Europe.

Practical creativity

Europe needs to take two key steps. First, it must be much more creative about financing ventures. What should matter is whether a policy works, rather than an ideological attachment to the market or public intervention. At least 15 schemes like University Challenge should bloom. Second, Europe needs to revisit the financing of pure research. A company such as AstraZeneca has the resources to undertake research that is so forward-thinking as to approach science fiction. Much more should be done to attract funding for curiosity-driven science on two grounds: businesses are part of the community and should contribute to it; and today's pure research is tomorrow's marketable product. A Europe-wide private-finance initiative for pure science (a UK scheme that brings together public activity with private finance and management expertise) could greatly change the entrepreneurial culture.

In the private sector, Europe needs a thriving venture-capital industry that genuinely aims to help the ambitious entrepreneur to create a business. The United Kingdom has made a start; Germany is developing fast from a low base; the Netherlands has an industry. None comes close to the scale found in the United States. Europe must put much more effort into attracting the high-tech entrepreneur, searching campuses for commercial ideas and putting together imaginative financing.

Universities can educate students, at all levels, to take business seriously. This practice flourishes in the United States but hardly exists in Europe. Universities also need to look at how they reward scientists. Of course they will continue to want them to teach and do research, but they should also give credit within the university for entrepreneurial activity. This is about more than money. Do an entrepreneurial scientist's intellectual peers recognize his or her contribution to business and society as worthwhile? There must be action by all parties to the deal — scientists, companies, venture capitalists, financial institutions and governments. The stakes are big, the prizes enormous. ■

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1. www.dti.gov.uk/ost/SETstats98/

2. *Risk Capital: A Key to Job Creation in the European Union* (European Commission, Brussels, 1998).

3. www.bvca.co.uk/BVCA/Welcome.html

4. *The Report of the Working Group on the Financing of Early Stage High Technology Companies* (UK Treasury, London, 1998).

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Ebb and flow in the seas of faith

Religion in the last century was forced to come to terms with scientific realities.

God's Funeral

by A. N. Wilson

Murray: 1999. 402 pp. £20

Hugh Montefiore

The author of this book, A. N. Wilson, is a prolific journalist, an acclaimed novelist and a brilliant biographer. He is also a person with deep religious impulses who studied theology at Oxford. In 1985 he published a book in which he affirmed the Christian religion to be "inescapably and irresistibly true". Six years later he produced a counterblast, *Against Religion*, wondering whether religion is the root of all evil, and dissociating himself from any formal organized religion. There followed books on *Jesus* (1992), where he holds that the Jesus of history was simply a Galilean holy man, and on *Paul* (1997), in which the apostle, if anyone, is the real founder of Christianity; both views, incidentally, held by earlier sceptics.

I mention these previous books because this latest work should be seen in the context of the swinging pendulum of his religious convictions. (The title *God's Funeral* is taken from a poem by the novelist Thomas Hardy, a man of natural religiosity who lost his faith in the face of pointless suffering.) The book sets out to "revisit the Victorian experience of faith and doubt", although it contains much more about doubt than faith — there is a whole chapter on Swinburne but one bare mention of Browning.

It contains vignettes, fascinatingly written, mostly of eminent Victorian doubters. Why did he write it? It is not a new subject. Victorian doubt has already been investigated by scholars. Is he using past history to help him resolve his agonizing dilemmas in the present?

"Is our personal religion," writes Wilson, "that which links us to the ultimate reality, or is it a final human fantasy, the most pathetic demonstration, in a spiritually empty, spacially limitless universe, of human aloneness? ... The way in which our nineteenth-century forebears confronted this, the most basic of questions, will always have implications and echoes in our generation."

The result of his survey is, perhaps surprisingly, that religion is the deepest kind of life, and he comments, at the book's conclusion, on the palpable and visible strength of "the Christian idea". George Romanes receives only a passing mention in the book, but I am reminded that in 1874 he published a defence of prayer, and in 1878 counterblasted with an attempt to disprove all arguments for God, but by 1883 had begun a slow and stormy return to faith.



Grief in the face of faith and doubt

This photograph of a figure of Christ is from the former Franciscan mission church of San Miguel, Huejotzingo, Puebla. It comes from *Mexican Churches* (Chronicle Books, \$18.95), a compilation of photographs of historic religious sanctuaries taken by Eliot Porter and Ellen Auerbach in 1955.

Romanes was a scientist who had concluded that Darwinism had disproved Christianity. That was a view untypical of most scientists of his day, which Darwin himself could not accept. Darwin, reserved about his own convictions, became an agnostic largely through the premature death of a beloved daughter. But just as Charles Lyell had shaken people by showing that the Earth was far older than the biblical calculations of its age, so Darwinism was deeply unsettling in an age when the Church taught that the words of Genesis are inerrant, and that species occurred through God's special creation. Wilson rightly points out that the Darwinian theory of evolution, through the mechanism of natural selection, removed the necessity of purpose in the discussion of natural history. (It was only later neo-Darwinism that removed the possibility of purpose.) Wilson

lends credence to the myth that Thomas Huxley, at the 1847 meeting of the British Association, won the argument over evolution with Bishop Wilberforce. It was Sir Joseph Hooker who won that: Huxley succeeded by his rhetoric.

Among the many freethinkers described are Edward Gibbon, who chronicled the less savoury aspects of Roman imperial Christianity; Thomas Carlyle, who believed that the idols of faith had to be destroyed but feared the consequence of their destruction; Auguste Comte who instituted a new Church complete with humanist saints; Karl Marx who believed that mankind is the product of economic forces; George Eliot (Mary Ann Evans) who believed God is inconceivable, immortality unbelievable and duty unconditional; Herbert Spencer, who held that science would be the instrument of perpetual progress; Edmund Gosse and Samuel Butler, who lost faith in a Heavenly Father when they lost faith in their earthly fathers; Thomas Huxley, who coined the word agnostic; Sigmund Freud, who believed that happiness lay in destroying the illusions of family life; Matthew Arnold, who preferred Hellenism to Hebraism and listened to "the melancholy long withdrawing roar" as the sea of faith receded; John Ruskin, an agnostic for most of his life, but nonetheless insistent on a religious dedication for members of his guild.

Incidentally, readers must prepare themselves here for Wilson's vagaries; for example, for George Bernard Shaw's "essential shallowness" and Wordsworth's fearfulness, who "followed his friend Samuel Taylor Coleridge's journey to rabid reactionary membership of the established Church".

Wilson's pen pictures are enlivened by glimpses into personal lives, making the book very readable. But it lacks sufficient contemporary background to make agnosticism fully comprehensible (few were outright atheists), and fails to contrast this with present-day presuppositions. For example, secularism is rampant today compared with the last century; while the argument from design, rightly abandoned in its last century form, is now based on the extraordinary "coincidences" in nature's constants which have made possible cosmic evolution.

The Victorian age was a time of great change, which always unsettles the mind and sets it at conflict with the heart. Brought up to believe that the Old and New Testaments were literally and verbally inspired, people were confronted with compelling evidence that they were not. The Gospels, hitherto immune to questioning, were now subject to rigorous literary and historical criticism. The

Church was unprepared for a rapidly growing urban proletariat. More people were being educated. Science was opening up a new world. Tractarianism emphasized the authority of the Church; and all the churches were solidly conservative.

In 1864 a collection of Anglican essays, *Essays and Reviews*, which questioned conservative views of scripture, was declared by a court of law to be consonant with the Church of England, yet over 10,000 clergy protested. Public opinion backed the deposition of Colenso, first Bishop of Natal, when he showed that the statistics of people and animals in the Pentateuch are absurd. Biblical criticism was not acceptable until the last decades of the century. As for the Roman Catholic Church, attempts to fit the ancient doctrines of the Church into modern dress were savagely suppressed as late as 1907. No wonder freethinkers experienced feelings of liberation.

When Wilson wrote that "the closing decades of the nineteenth century were the true era of 'the death of god'", he must have omitted to consult records. In 1894 less than 18% of all weddings were civil marriages. Three out of four children went to Sunday school. Despite much urban indifference, baptisms and confirmations were still rising, both relatively to the population and absolutely, and so was the number of clergy. Wilson is writing about the intellectual elite. The real slump came later.

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Managing a crisis out of context

Water Wars: Is the World's Water Running Out?

by Marq de Villiers

Weidenfeld & Nicolson: 1999. 413 pp. £20

Robert Gottlieb

A few years back, the governor's office in Alaska sent me a poster. It promotes the "Alaska-California Sub-Oceanic Fresh Water Transport System", which is defined as a "2,000+ mile undersea aquaduct [sic] transporting pure Alaskan water from the abundant rivers of Southeastern Alaska to the arid Southwestern United States". The bottom of the poster has this quote from former Alaska governor and US interior secretary Walter J. Hickel: "Big projects define a civilization. So why war — why not big projects?"

I keep this poster on the wall near my office for my students to see, not because I assume that the project is likely to occur — it isn't — but because it captures some of the



A basic human right? One billion people have no access to clean water.

absurdity associated with how discussions of water availability have long been framed. Even more, it describes the difficulties, if not the crisis, affecting the long-standing water-management strategy of somehow transporting water from where it's plentiful to where it's not, regardless of the environmental, social and economic consequences of such an undertaking.

It is this notion of crisis in the management and availability of water, or what he calls the emerging "water wars", that is the central theme of Marq de Villiers' book. De Villiers provides a regional or country-by-country synopsis — a sequence of water stories — about the nature of those wars. And although the book's title suggests a focus on water availability — that is, how much water is available on a per capita basis in a particular location — other core issues are also examined. These include: issues of water quality associated with the depletion and contamination of groundwater and surface-water sources; political issues concerning the control of water sources, particularly interstate and regional conflicts; environmental issues associated with the degradation of water sources, such as those caused by building dams or channelizing rivers; economic issues about the costs involved in making water available, as well as the cost of water for the individual user; food and agricultural issues concerned with the increasing reliance on irrigated production; and public-health issues associated with sanitation and drinking water.

Each of these issues is formidable in its own right, let alone in combination. A recent

report by the World Commission on Water for the 21st Century, for example, identified the phenomenally high prices that the poor in less developed countries pay for their water, even as the quality of that water has become increasingly contaminated.

The US-based Pacific Institute for Studies in Development, Environment and Security has estimated that more than 1 billion people have no access to clean drinking water, and nearly 3 billion have no access to sanitation services. And Population Action International has further estimated that, in 1997, 436 million people were living in conditions of "water stress and water scarcity". Even in places like England and the United States, inappropriate water management and land use have led to a significant groundwater overdraft in places like the Thames valley and the Ogallala aquifer which stretches from Texas to Nebraska. And, as De Villiers demonstrates in his survey of the regional battlefields over water, the control and use of such ancient water sources as the Tigris and Euphrates, the Jordan river and the Danube are capable of increasing or renewing hostilities between states.

De Villiers' study is best when he captures the details of the stresses involved in controlling and using water, as seen in the political conflicts that have sprung up in nearly every part of the world. His recommendations for possible strategies, however, are less clear. If the problem of availability is due more to unequal and wasteful allocation and distribution than to insufficiency of water (and De Villiers seems to argue both ways), then strategies that focus on how we use water may be most compelling. Similarly, De Villiers debunks some of the schemes for importing water from distant places (such as the Alaskan transport of water via pipeline to the southwestern United States), and seems cautious about utilizing expensive and energy-intensive technologies such as desalination. But he's not prepared to rule out importing water or employing technologies as core solutions.

What De Villiers' analysis also lacks is a discussion of the significance of water in its social and cultural as well as environmental settings, an issue alluded to only when De Villiers describes how his father, who was a farmer, used the water available to him. If water access and availability are core issues of daily life, as De Villiers argues, that provides a starting point for looking at how water use and availability are incorporated into different cultures and their significance for different communities. It also suggests how access to water constitutes a basic human right. And by identifying what University of California professor Helen Ingram has called water's community value as opposed to its commodity value, a more focused framework for identifying solutions can be developed.

As a collection of stories about the range

of problems associated with water, *Water Wars* guides us well through the arenas of conflict. As an evaluation and analysis of the strategies for change, a greater focus on the context of the problems is needed. ■

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Astronomical expansions

The American Astronomical Society's First Century

edited by David H. DeVorkin
AAS: 1999. 350 pp. \$45.95

Jayant V. Narlikar

The second of the three founding meetings of the American Astronomical Society (AAS) was held in the director's drawing-room at the Harvard College Observatory in 1898. Today's AAS meetings can attract more than 1,000 participants, with the venue a large hotel with auditoria for plenary and multiple sessions. This transformation is well documented in *The American Astronomical Society's First Century*, which is edited by the Smithsonian historian of science David DeVorkin, and has contributions from 28 contemporary astronomers, historians of science and science administrators.

The book is replete with fascinating anecdotes: from the evolution of the society in those early meetings, the cautious welcome accorded to amateurs and the early controversy between the roles of astronomy and astrophysics (which, in 1914, finally resulted

in the present name of the society), to its spread across the northern border to include Canadian members. It is particularly sobering to read the account by Susan Simkin of Margaret Burbidge's rejection of the society's Cannon prize (for women astronomers) on the grounds that it was discriminatory. It highlights how difficult it was (and still is) for women to sustain careers in astronomy.

Predictably, the Second World War was a watershed, after which organized scientific research was to take charge of astronomy. The society provided an excellent platform for astronomers to hold dialogue with the government, particularly with the Office of Naval Research for supporting astronomy projects. This office, and later the National Science Foundation, had a strong influence on the development of astronomy research in the United States. One article, by Joel Stebbins, written in 1947 around the 50-year landmark of the society, has been reproduced.

The demographic account of the society shows the steep rise in AAS membership, which started in 1960 and continues today. The society grew as astronomy itself expanded, and the structure of the AAS meetings had to change. The moment of truth arrived with the society's 91st meeting in 1953, when parallel sessions became essential. Whereas an earlier decision had stressed that simultaneous sessions would be allowed "only if absolutely necessary, and as a last resort", today's meetings have multiple sessions and poster sessions, and large crowds that make old-timers like Bill Baum lament: "I don't know any of these people". Indeed, in today's world of 'Write proposals or perish', one may wistfully look back to the early days of relaxed informality. This development is a pity, because the vastly increased cost of frontier research encourages organized science, with very little scope for individual geniuses and mavericks.

The inexorable march towards organized professionalism continued, with the society needing an executive office which it later moved to Washington. The society's research mouthpieces, the *Astronomical Journal* and the *Astrophysical Journal*, grew in stature and size. In 1971, S. Chandrasekhar, as editor, ran the *Astrophysical Journal* with one associate editor, one assistant, a production person and a copy-editor, a far cry from the army of manpower needed today.

The book itself shows professionalism in its planning, and for the reader interested in archival statistics and in how a group of scientists with a common interest organized and reorganized themselves to best deal with rapidly changing conditions, there is much useful material. On two counts, however, I was disappointed. The book could have contained some account of how astronomers evolved their view of the cosmos through



Sisters in science: Caroline Furness (left) and Anne Cannon, members of the AAS in 1930.

debates and discoveries. How did the society receive the Shapley–Curtis debate? What was its attitude when new observations placed the Galactic Centre away from the Solar System? How did it react to the discovery of quasars, or to the COBE satellite? Those must have been exciting times, when astronomical history was being made, and readers in the twenty-first century would have enjoyed glimpses of it. Similarly, a century of interactions involving famous personalities and famous observing instruments must have generated many memorable anecdotes, like the Yerkes disaster of 29 May 1897 described early in the book by Donald Osterbrock, when the floor of the newly built Yerkes telescope collapsed, fortunately without injuring anyone. However, not many such anecdotes follow. ■

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Perceiving confluent disciplines

Vision Science: Photons to Phenomenology

by Stephen E. Palmer

MIT Press: 1999. 801 pp. \$70, £48.95

Richard Gregory

This is an impressive, well-produced and easy-to-read textbook of visual science, even though it is quite technical in places. It is enlivened with historical asides and has underlying philosophical themes, although its main thrust is to present the current experimental understanding of how we see.



The 1897 meeting at the Yerkes Observatory, where talks on forming the society began.

The philosophical approach follows the nineteenth-century polymath Hermann von Helmholtz, who famously conceived perceptions as unconscious influences created from quite limited sensory data. They also originate from knowledge of the world of objects, and are generated from various rules. In practice, these are assumptions, as knowledge from the past may not be appropriate to the present situation, and the rules do not always apply. Thus, converging lines often represent parallel lines receding in depth, but they may simply be converging lines normal to the observer. This illustrates the paradox of pictures, and is an important way in to thinking about cognitive errors of perception — the most suggestive phenomena of illusions.

This book is richly influenced by the burgeoning emphasis on the importance of knowledge for interpreting visual data. In other words, the present cognitive approach to what used to be thought of as a direct, quite simple awareness of the external world. The study of perception has become far more interesting — it embraces ideas from information technology and even from the philosophy of science (for perceptions are quite like predictive hypotheses of science), as well as the remarkable physiology and anatomy of the visual nervous system.

A student taking this book seriously will learn a great deal about perception of form, objects, colours and movement. One great merit is its clarity and conciseness of expression, despite its considerable bulk. The illustrations are well chosen and not merely for show; they enhance the meaning of the text without being distracting. This book can be thoroughly recommended as a text for students willing to look at perception from a broad basis in considerable detail, while leaving intriguing questions of how tantalizingly experience is related to brain function before the curious mental eye. ■

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Prosaic poetics

The Poetics of Natural History: From John Bartram to William James

by Christoph Irmscher

Rutgers University Press/Eurospan: 1999.

320 pp. \$42/£37.50

Juliet Clutton-Brock

In giving his book the title *The Poetics of Natural History*, Christoph Irmscher's aim seems to be to intrigue and impress literary historians with the writings of early American naturalists. He claims that the book is about the daydreams of American natural-

ists from 1730 to 1868. These 'daydreams' seem like all too real experiences to me — the financial difficulties of John Bartram in trying to extract payment from his English patron, the stench of rotting bird carcasses that John Audubon had to paint, and his description of the slaughter of thousands upon thousands of passenger pigeons, piled up in great heaps to be fed on by wild carnivores as well as by domestic hogs.

Beginning with Bartram and his son William, who for 40 years collected plants for export to the English gardener Peter Collinson, the book centres on the lives and works of Charles Willson Peale who founded the Philadelphia Museum, the great showman Phineas T. Barnum, the painter Audubon and the zoologist Louis Agassiz with his student William James.

As befits the invaders of a 'new' continent, there is a rawness in the behaviour and attitudes of the early American collectors that matches that of the nineteenth-century big-game hunters of India and Africa. All living things were created for the benefit of man, and anything that moved was a target for the gun. Life was tough and, undoubtedly, these men had much to contend with. It is therefore not surprising that they placed more emphasis on the dangers of the rattlesnake, to which Irmscher devotes a whole chapter, than on the 'peeps at nature' that entertained the occupants of Victorian drawing-rooms in England.

In a chapter called "Collecting human nature", Irmscher relates the complicated story of the showman Barnum in whose American Museum a huge and extraordinary assemblage of objects, curiosities, fakes, live animals and disabled humans was exhibited.

In 1868, after two catastrophic fires, Barnum shifted his interests to a circus that he moved from place to place in a railway train, and it was then that he purchased the famous African elephant Jumbo from the London Zoo. Jumbo barely receives a mention in the book, but he was the most notorious and perhaps one of the saddest animals of the Victorian era. He became unmanageable at the zoo and was sold to Barnum for £2,000 (see W. P. Jolly's *Jumbo*; Constable). The initial cause of Jumbo's ill temper can be seen in the plaster cast of his upper jaw, which is in the collections of The Natural History Museum, London, and shows that he must have suffered excruciating pain from severely compacted molar teeth.

Peale was different from Barnum, and his huge collections were more in line with those of the great European naturalists such as Hans Sloane. He had "a large collection of minerals, 4,000 species of insects, 90 species of stuffed quadrupeds, and over 700 mounted birds", and all were ceaselessly expanded. Peale was also an accomplished

painter, and his self-portrait, "The artist in his museum", was a central exhibit.

Peale, like most people of his day, had a total belief in the Creation and the biblical flood, and he "never tired of searching for an antediluvian (but still fairly recent) America". Specimens like the excavated skeleton of an American mammoth were proudly exhibited as evidence of destruction by the deluge.

An exception to these self-taught collectors and the illustrator Audubon was Agassiz, the highly educated Swiss zoologist who had studied under Georges Cuvier, and who emigrated to Harvard in 1846, where he founded the Agassiz Museum with his collections in 1860. Agassiz was certainly a great naturalist and, like T. H. Huxley, a brilliant public speaker. However, unlike Huxley, he was an anti-evolutionist who was not won over by the complimentary copy of the *Origin of Species* that Charles Darwin sent to him in 1859.

I can see very little in this book that could be described as "poetic", or that can stand on its own as literature, as do Gilbert White's *The Natural History and Antiquities of Selborne* (1789; Intercept Scientific) and Henry David Thoreau's *Walden* (1854; Everyman). The book also lacks the charm and smooth writing of Lynn Barber's *The Heyday of Natural History 1820–1870* (Jonathan Cape). However, the accounts of the lives, attitudes and collections of the early naturalists in North America provide a valuable addition to the history of the early European colonists as well as to that of natural history in the New World.

It is easy to see the early naturalists either as brash entertainers or as quaint old people peering at pond water through ineffective brass microscopes. These people were limited by the technology available to them, but they were as serious in their efforts to understand and promulgate the wonders of nature as are the makers of wild-life programmes today. I wonder if, in 100 years from now, David Attenborough's film of his first encounter with a wild gorilla (which will most probably be extinct by then) will be likened to Audubon's description of the flights of passenger pigeons. ■

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Natural history then and now **Empire's Nature: Mark Catesby's New World Vision**

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A fistful of wishful thinking

The theory that settlement would bring rain turned to dust — like the fields.

Daniel J. Kevles

Rain follows the plough, people said knowingly in late-nineteenth-century America. The phrase was promulgated in a book published in 1881 by one Charles Dana Wilber, a land speculator who was *au courant* with the climatology of his day. It distilled the theories of some prominent Earth scientists that settlement and cultivation, especially the planting of trees, would transform arid regions, notably the Great Plains beyond the hundredth meridian, into fertile, loamy expanses bathed in rain, like the agriculturally fecund Mississippi Valley.

Publications by European scientists on forests and rainfall formed a major source of theorizing. Their ideas were brought to the attention of US readers by George Perkins Marsh in his remarkable treatise of 1864, *Man and Nature; or, Physical Geography as Modified by Human Action*. Marsh, a polymathic businessman, diplomat and writer, was a New Englander appalled by the deforestation of his native region and an advocate of planting and preserving trees. While Marsh faithfully acknowledged that the rain-making power of trees was “not entirely free from doubt,” he reported that the balance of European scientific judgement believed that forests exercise a “certain and undisputed” meteorological influence.

That was enough for Ferdinand V. Hayden, a geologist and explorer of the American west and, beginning in the late 1860s, the head of the US Geological Survey of the Territories. In 1867, in his agency’s first report, he announced that the timber increase arising with settlement had already elevated rainfall in Nebraska, and that similar change would “extend across the dry belt to the foot of the Rocky Mountains as the settlements extend and the forest-trees are planted in proper quantities”. Hayden’s assistants at the survey enlarged on the claim, declaring that rainfall increased not just with trees, but with ploughing, and possibly even with the laying of telegraph lines and railroad tracks.

During the 1870s, as settlement spread westward across the Great Plains, rainfall happened to increase, giving a seeming plausibility to the arguments of Hayden and his colleagues. But the geologist John Wesley Powell was unconvinced. “In what manner rainfall could be affected through the cultivation of the land, building of railroads, telegraph lines, etc., has not been shown,” he wrote in 1878, in a *Report on the Lands of the Arid Region of the United States*.

Powell argued that rainfall in the arid

The episode would not be the last to combine contested theories of climate with passionate convictions of political economy.

region could never sustain agriculture based on the traditional 160-acre homestead. That system of apportionment had to give way to one that allowed for much larger sections suitable for dry-land ranching and irrigation farming. Their contours would be shaped by their access to water, and their size — up to 2,500 acres — would be determined by the use to which the land could be put. Powell’s ideas were the basis of legislation for the arid region introduced in Congress in February 1879, and commended by easterners of all political persuasions as wise, realistic, and scientifically authoritative.

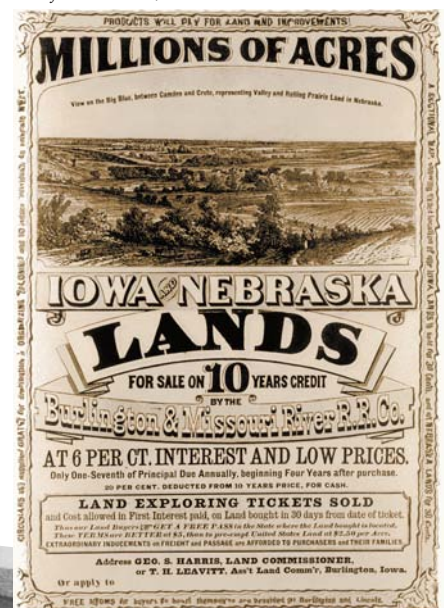
But representatives from the west derided and killed the measure. To them, ambitious inhabitants of a developing region, Powell’s initiative resembled a kind of environmental colonialism. Theorists had pronounced the homestead system dead before, cried the delegate from the Montana Territory, yet settlers had gone west and, “practical men” all, had “seen the capabilities of this land which had escaped the notice of our scientists and statesmen”. A congressman from Kansas admonished scientists who explored the west: “If you please, while you are out there acting in the interest of science and in the interest of professional bug-hunting, leave the settlers upon our frontier alone.”



Land up for grabs: but many homesteaders were ruined when rain stopped ‘following the plough’.

Powell nevertheless had the science essentially right. According to modern views, trees do bear some relationship to the moisture content of the air in their neighbourhood, but not to rainfall. On the Plains during the 1880s, rain stopped ‘following the plough’, ruining numerous settlers; irrigation would prove indispensable to farming in the region. But, at the height of the dispute, opposition to the kind of land reform advanced by Powell had on its side the scientific theorizing of Hayden and its links to the interests of small farmers by Wilber, who characterized the proposed departure from the “equable” 160-acre homestead system as the work of a “powerful ring” in Washington bent on serving the “aristocratic tastes” of the cattle barons. The episode would not be the last to combine contested theories of climate with passionate convictions of political economy. □

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Cracking anaerobic bacteria

John Parkes

Hydrocarbons such as hexadecane have seemed resistant to bacterial decay to methane. Not so, it turns out, but this bacterial hydrocarbon 'cracking' process is very slow.

At any one moment, some ten billion tons of particulate organic matter is sinking down through the world's oceans¹. This organic matter stimulates microbial activity to such an extent that only a small proportion of it — less than 0.5% — survives the long descent through the water column to become buried in sedimentary layers². Nonetheless, over geological time, the cumulative effect is huge; sedimentary rocks constitute the world's largest reservoir of organic carbon and they are, of course, a source for fossil fuel.

But how does even this 0.5% or so of organic matter survive microbial degradation within sediments? Several different mechanisms have been proposed, such as increased phytoplankton production, selective preservation of naturally decay-resistant compounds, production of resistant polymers during decay, and preservation by binding to inorganic particles (see ref. 2 for review). Another factor is the preferential use, and hence removal, of the most efficient respiratory compounds (oxygen, nitrate, iron and sulphate) by bacteria near the sediment surface. For instance, aerobic bacteria near the sediment surface will rapidly use oxygen, resulting in the advent of nitrate-metabolizing bacteria, and so on. In consequence, bacteria deeper in the sediment have to use less efficient respiratory compounds and so obtain less energy from the degradation of organic matter, which may contribute to the preservation of certain organic compounds.

Hydrocarbons, produced by a range of organisms both marine and terrestrial, are examples of decay-resistant compounds, especially in the absence of oxygen. This characteristic would explain why hydrocarbons in deep oil reservoirs, without much flow of water to supply oxygen, survive undegraded. Only in the past ten years has any anaerobic degradation of hydrocarbons been shown to occur, and the organisms concerned include denitrifying, ferric-iron-reducing and sulphate-reducing bacteria³. The final anaerobic decay process, methanogenesis — production of methane (CH₄) — has long been known through the emanations of marsh gas from wet environments such as ponds (Fig. 1). But, in terms of hydrocarbons, it was thought to be restricted to degradation of monoaromatic and unsaturated compounds (benzene and hexadecene, for example). Saturated hydrocar-

bons, such as hexadecane, seemed resistant to decay by methanogenesis⁴.

Now we must think again, for on page 266 of this issue Zengler *et al.*⁵ report laboratory experiments in which 'enrichment cultures' of bacteria are shown to degrade hexadecane (and pentadecane) completely to CH₄ and CO₂. The fact that this process is very slow — four months to start CH₄ formation, and incubation for over two years to obtain enough CH₄ to work out the stoichiometry for the overall reaction — may explain why these bacteria have been overlooked in the past.

Zengler *et al.* find that the degradation of hexadecane is a team effort involving several

species of interacting bacteria. This is a common phenomenon in anaerobic bacterial processes, but it does of course complicate investigation of the reactions concerned. For example, previous reports of methanogenic degradation of hydrocarbons have also suggested that mixed bacterial populations are involved, but that they include both aerobes and anaerobes⁶. In this case, aerobic oxidation of hydrocarbons produced acetate and higher fatty acids, plus other compounds that were fermented to H_2 and CO_2 . The acetate and H_2 were then utilized by methanogens for CH_4 formation.

Zengler and colleagues' proposed scheme

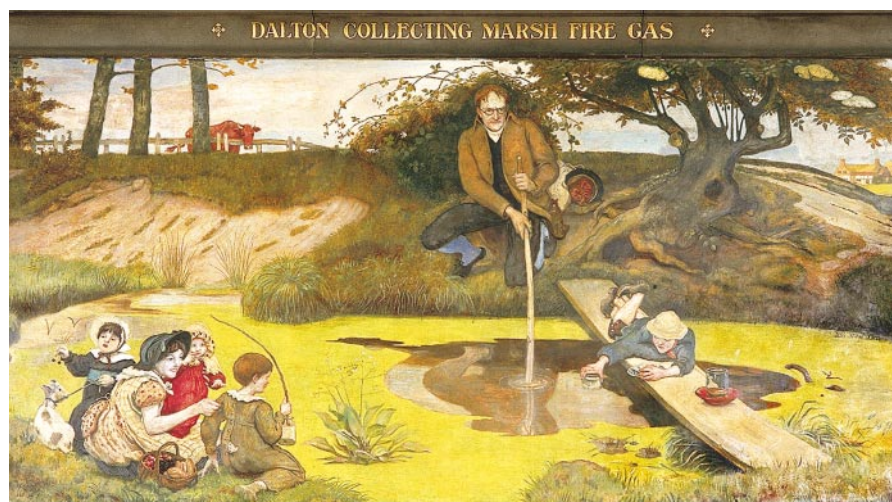


Figure 1 Natural gas and the chemist. Here John Dalton (1766–1844) is depicted collecting marsh gas by poking a stick into pond sediments; the artist is Ford Madox Brown. Marsh gas (methane, CH_4 , the simplest alkane) is the main component of natural gas. As shown by the work of Zengler *et al.*⁵, summarized in Fig. 2, new aspects of this economically important bacterial process are still being revealed.

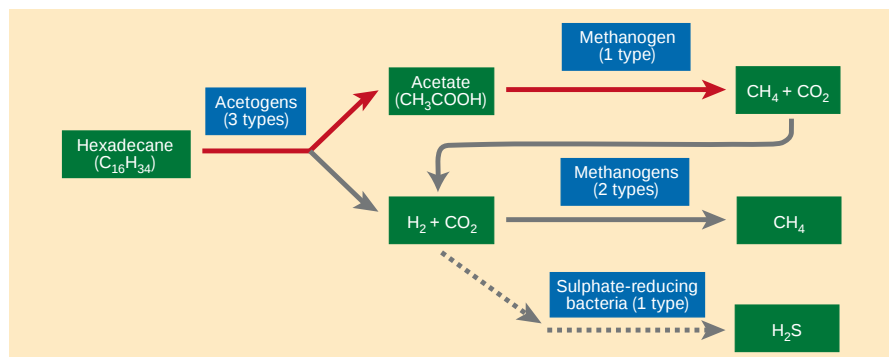


Figure 2 Making methane from hexadecane — the interacting team of seven bacteria that ‘cracks’ hexadecane anaerobically to CH_4 gas. The bacterial types are in blue; substrates and products are in green; red shows the dominant pathway.

is similar except that, remarkably, only anaerobes are present in their bacterial community. The results will be contentious, as it is difficult to exclude oxygen during sampling and long-term incubations of this kind. However, Zengler *et al.* found that no growth occurred when the culture was incubated in the presence of oxygen, so demonstrating the absence of aerobic bacteria, whereas growth was unaffected when sealed culture bottles were incubated inside an anaerobic jar to completely exclude oxygen.

The hydrocarbon degraders seem to be acetogenic bacteria that initially metabolize hexadecane to acetate and H_2 (Fig. 2). These acetogens are known as syntrophs because they require the presence of other anaerobes for their metabolism to be effective: they can obtain energy only from hydrocarbon degradation if the metabolic waste products are continuously removed and kept at very low concentrations. This is where the methanogens come in. There are two different types of them present in Zengler and colleagues' bacterial enrichments. One uses the acetate and the other uses the H_2 , and they keep the concentrations of each low enough to enable the anaerobic degradation of hexadecane to proceed.

Zengler *et al.* use molecular genetic techniques to confirm the composition of this bacterial community (see their Fig. 2 on page 268). It includes three closely related syntrophic bacteria similar to cultured *Syntrophus* bacteria and, interestingly, a bacterial gene sequence akin to that from a hydrocarbon-contaminated aquifer⁷. Gene sequences related to both acetate- and H_2/CO_2 -utilizing methanogens are also present.

But here the plot thickens, as there is also a gene sequence closely related to *Desulfovibrio*-type sulphate-reducing bacteria, and the culture grows better in the presence of a small amount of sulphate (about 2 mM). So, in nature, both sulphate reduction and methanogenesis may be required for even this slow process to occur. The requirement for low sulphate concentrations may restrict the process to specific environments because higher concentrations might result in sulphate reduction becoming the dominant reaction⁸, with toxic and corrosive H_2S being the end product.

There are, however, indications that CH_4 production from hydrocarbons and other seemingly decay-resistant organic compounds does occur in the environment: for example, in the San Juan coal formation (Colorado and New Mexico), the most prolific coalbed gas basin in the world⁹; in some oil reservoirs¹⁰; and even from ancient organic matter in limestone rocks of the Florida escarpment¹¹. The process may also help to explain the continued presence of bacteria in deep and ancient sedimentary formations^{12,13}, and it is of potential importance for the petroleum industry. If the large

amounts of hydrocarbons remaining in reservoirs after normal oil extraction could be converted to CH_4 , then a biogas reservoir could be created and so greatly increase the recovery of fossil fuels. ■

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Microbiology

Salmonella strikes a balance

Michael S. Donnenberg

In landmark studies done more than 30 years ago, Takeuchi¹ described the invasion of intestinal cells by *Salmonella* at an ultrastructural level. His careful observations detailed not only the dramatic alterations in a cell's architecture that accompany bacterial entry, but also the equally striking restoration of the cell's shape once the bacterium is inside. Although considerable attention has been paid to the molecular mechanisms that allow some bacteria to invade cells, until now the process that allows cells to recover has received scant attention. But on page 293 of this issue, Fu and Galán² report the characterization of a bacterial protein that puts the brakes on signals leading to disruption of cellular architecture. This allows the host cell to recover its form—a change that benefits the microbe as well as the host.

Entry of *Salmonella* into the epithelial cells that line the gastrointestinal tract is an essential early step in severe illness. To force cells that do not normally take up bacteria to engulf them, *Salmonella* injects a protein into the host cell that switches on critical regulators of host cell shape. The bacteria are taken up during the ensuing convulsions of the cell membrane. Fu and Galán have now found that *Salmonella* also injects a second protein into host cells that increases the rate at which these same regulatory proteins switch off. Thus, both the disruption and restoration of cellular architecture are under bacterial control. By producing two effector molecules that target the same cellular switches but act in opposite directions, *Salmonella* seems to have mastered the yin and yang of bacterial invasion.

Cellular architecture is maintained by an assortment of structural proteins, including actin, which form a dynamic cytoskeleton. A family of small GTPases, among them Rho, Rac and Cdc42, help to regulate the actin

cytoskeleton in response to external stimuli³. The activated forms of these molecules are bound to GTP, whereas the inactive forms are bound to GDP. Activation of these (and similar) GTPases is facilitated by guanine-nucleotide-exchange factors, which increase

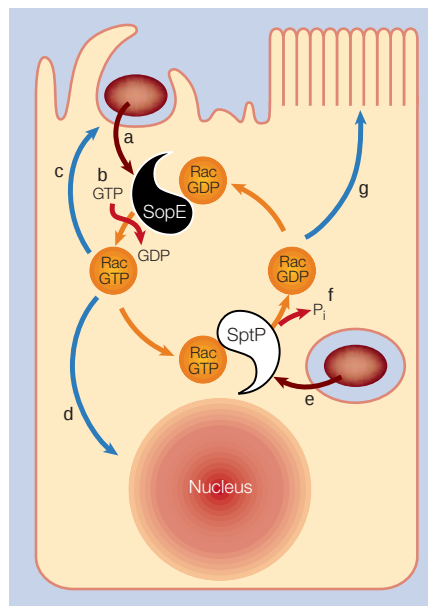


Figure 1 The yin and yang of bacterial invasion. Fu and Galán² have shown that the bacterium *Salmonella* alters cell shape by activating and deactivating small GTPases. a, The bacterial SopE protein is delivered to the host cytoplasm where it binds to Rac. b, SopE acts as a guanine-nucleotide-exchange factor to favour the GTP-bound, activated form of Rac. c, Activated Rac stimulates membrane ruffling, which results in bacterial uptake, and (d) activates nuclear factors that affect gene transcription. e, The bacterial SptP protein, also delivered to the host cytoplasm, binds preferentially to GTP-bound Rac and (f) enhances its GTPase activity. g, Cell shape is restored in the absence of active Rac.

the rate at which GDP dissociates from the molecules and GTP associates with them. Conversely, GTPase-activating proteins accelerate the rate at which these proteins hydrolyse GTP to GDP, and so facilitate inactivation⁴.

Once activated, Rho, Rac and Cdc42 direct the assembly of actin monomers into dynamic filaments that can serve a variety of purposes. The formation of actin stress fibres, for example, is stimulated by activated Rho and helps to maintain cell shape. By contrast, the formation of actin-rich lamellipodia and filopodia, stimulated by activated Rac and Cdc42, respectively, allows cells to form membrane ruffles and protrusions. Rho, Rac and Cdc42 also affect transcription of cellular response genes by activating other factors including NF- κ B, mitogen-activated protein kinase and the *c*-Jun amino-terminal kinase.

Earlier studies had shown that *Salmonella* exploits Rac and Cdc42 to alter the structure and function of the cytoskeleton. The bacteria produce a protein known as SopE, which is injected into the cytoplasm of the host cell through a so-called type-III secretion apparatus. The SopE protein binds to Rac and Cdc42, and acts as a guanine-nucleotide-exchange factor to tip the balance towards the activated, GTP-bound forms of these signalling molecules⁵. The activated GTPases stimulate the formation of membrane ruffles that engulf the bacteria, allowing them to reach their preferred site in specialized intracellular vacuoles. Earlier studies had also identified a protein known as SptP, which is also injected by *Salmonella* into host cells via the type-III secretion system⁶. The SptP protein seemed intriguing because of its modular structure (which includes a tyrosine phosphatase domain), and because it induces cytoskeletal changes when microinjected into cells. But its function in infection was unclear because a mutant unable to produce SptP has no defect in invasion.

Fu and Galán² now resolve this paradox. They report that cells infected with a mutant strain of *Salmonella* that cannot express SptP continue to show evidence of cytoskeletal disruption hours after infection — long after cells infected with wild-type *Salmonella* recover their shape. They found that simultaneous microinjection of cells with both purified SptP and SopE does not alter the cytoskeleton. But microinjection of either protein alone has dramatic effects. The authors also showed that SptP binds preferentially to the GTP-bound form of Rac, and that SptP stimulates the GTPase activity of Rac and Cdc42. In other words, it acts as a GTPase-activating protein. They then went on to identify a sequence motif present in both SptP and eukaryotic GTPase-activating proteins, and showed that if they mutated a critical arginine

residue in this region they ended up with a protein that can bind Rac but cannot activate its GTPase activity. A strain of *Salmonella* that produces this mutant form of SptP behaves just like a strain that produces no SptP at all. So, *Salmonella* interacts with GTPases that regulate actin structure and function at two points, both to activate them and to deactivate them (Fig. 1).

The authors suggest that expression or delivery of SopE and SptP must be controlled in a way that allows the proper sequence of events to unfold during *Salmonella* infection. More work needs to be done to appreciate this finely coordinated balance. Given that SptP's tyrosine phosphatase activity is not required for its effects on the cytoskeleton, SptP probably has other, as-yet-unidentified functions. And the fact that other bacterial pathogens express molecules resembling SptP indicates that similar strategies may be widespread, and that they could

potentially be exploited for therapeutic purposes.

The results of these studies should also stimulate general interest in an understudied aspect of infectious diseases. With all the attention paid to virulence factors that injure the host, the mechanisms that limit this damage remain all but ignored. Conceivably, a broad array of microbial 'regressins' might exist to ameliorate the havoc wrought by their 'aggressins'. After all, death of the host rarely serves the microbe. ■

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Cosmology

Universal peekaboo

Adam G. Riess

Near the end of the millennium, new observations and analyses are suggesting answers to some of the most profound and ageless questions. What is the extent of the Universe? How old is it? What is its shape? What is its ultimate fate? The answers to these questions have come from philosophers and religious leaders, but in the realm of experimental science answers are now coming from exploding stars and the gentle tug of gravity. Recent astronomical observations suggest that the Universe may be influenced by a positive 'cosmological constant' that affects its evolution. On page 252 of this issue¹, Zehavi and Dekel present a synthesis of several observations that tightens the case for a positive cosmological constant. The likely implications for the Universe of a positive cosmological constant are eternal, accelerating expansion.

This is an unusual moment in the history of cosmology, in which we are starting to look at the evolution of our Universe from an empirical rather than a theoretical point of view. Observations of astrophysical phenomena are providing meaningful constraints on the most elusive cosmological parameters, Ω_m and Ω_Λ , which together determine the energy density of the entire Universe (Ω_m measures the energy density found in 'normal' gravitating matter, such as galaxies and black holes; Ω_Λ describes the energy density found in exotic 'vacuum energy', better known as the cosmological constant). In normalized units, a sum of one for all energy densities in the Universe is the

exact amount required to flatten the fabric of space-time. Specific regions of the two-dimensional parameter space defined by Ω_m and Ω_Λ (Fig. 1, overleaf) imply simple answers to the previously mentioned 'big questions'.

The difficulty in modern observational cosmology is that we do not know of a single controlled experiment that would reveal the individual values of the cosmological parameters. Rather, cosmologists must coax nature into revealing the values of the parameters by combining data from ongoing astrophysical activity. Although data from different techniques will constrain a different mix of the cosmological parameters, we can combine such observations in the hope that they will converge on a single set of values. This has led to the birth of 'peekaboo' cosmology, in which cosmologists overlay independent constraints on the allowed cosmological parameter space until only a narrow space or window remains through which we can peek to find answers to our questions about the Universe^{2–6}.

A narrow region of this parameter space permitting a positive cosmological constant has recently been identified by observations of distant, exploding white-dwarf stars, called type Ia supernovae (SNe Ia)^{7,8}. By observing the apparent brightness of high redshift (or distant) SNe Ia, the High-*z* Supernova Team^{7,9} and the Supernova Cosmology Project⁸ found SNe Ia to be surprisingly dim for their redshifts, implying that a positive cosmological constant has been accelerating the expansion

of the Universe since the Big Bang. Yet because gravitating matter and a positive cosmological constant pull and push the expansion of the Universe in opposite ways, the data from SNe Ia alone still allow for many combinations of Ω_m and Ω_Λ .

Another approach derives a constraint on a different mix of the cosmological parameters by quantifying the degree to which local mass in different parts of the Universe tugs on nearby galaxies. Although most of the apparent motion of galaxies results from the expansion of the Universe, some excess or 'peculiar' velocity arises from the gravitational pull of nearby mass concentrations¹⁰. Zehavi and Dekel¹ obtained limits on Ω_m of the Universe by using observations of these peculiar velocities. These measurements favour $\Omega_m \ll 1$, which is a notable result from researchers who once provided the strongest evidence in support of a Universe flattened by mass (that is, $\Omega_m = 1$)¹¹.

The area of parameter space highlighted by combining these peculiar velocity results with the supernova results is seductively close to the theoretical preference for a geo-

metrically flat Universe. Yet, the danger of blindly combining (and trusting) the results of different measurements is to lose sight of the systematic uncertainties involved in individual techniques. For the peculiar velocity methods, if one or more assumptions are not valid (for example, that the initial mass fluctuations are normally distributed) then the implications may not be correct. Similar challenges face the conclusions drawn from supernovae data, such as the possibility of supernova evolution^{12,13} or exotic intergalactic dust, which could scatter light from supernovae making them appear further away than they are¹⁴.

By pushing the observations of supernovae to greater distances (and therefore further back in time) it should be possible to conclusively confirm or refute the initial indications that we are living in an accelerating Universe. We expect a younger, smaller Universe to be denser and to experience greater deceleration from gravity, so the apparent brightness of supernovae should decrease more slowly with distance than is likely to occur under the influence of systematic effects.

Finally, measurements of tiny ripples in the radiation left over from the hot Big Bang — the cosmic microwave background — are beginning to point towards a Universe which is geometrically flat. These first hints will soon be surpassed by data from the MAP and Planck satellites due early next year. By combining enough independent constraints it should be possible to arrive at a set of cosmological parameters that is not vulnerable to systematic uncertainties in any single measurement. Although some cosmologists have voiced concern that the recent studies imply that we live at a special time — that is, shortly after the transition from a decelerating to an accelerating Universe — can there be any time more special than when we first begin to learn the answers to our Universal questions?

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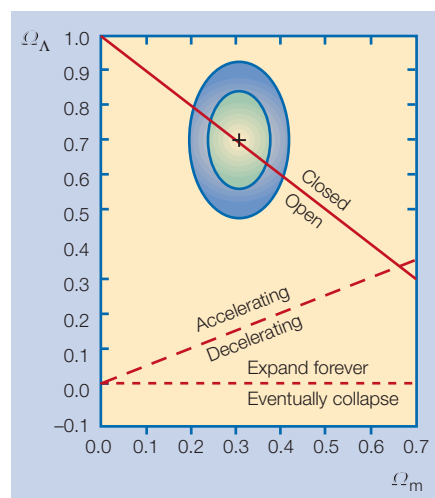


Figure 1 Window on the Universe. To discover the values of the parameters Ω_m (mass density) and Ω_Λ (cosmological constant) that define the evolution of the Universe, cosmologists overlay data from different astrophysical techniques to create a window in the allowed parameter space. Zehavi and Dekel¹ have combined data from studies of supernova and peculiar velocities to strengthen the case for a positive cosmological constant. The results of a similar study¹⁵ using nine independent astrophysical measures are shown here. The cross marks their best guess, where $\Omega_m = 0.3$ and $\Omega_\Lambda = 0.7$, and the contours represent relative confidence limits of 68% and 98%. The solid line corresponds to a flat Universe ($\Omega_m + \Omega_\Lambda = 1$), separating an open Universe from a closed one. The dotted line ($\Omega_\Lambda = 0$) separates a Universe that will expand forever from one that will eventually collapse in the Big Crunch, whereas the dashed line separates a Universe with an expansion rate that is decelerating from one that is accelerating.



100 YEARS AGO

One result of the rapid growth of seismology is the suggestion of Dr. Mario Baratta that provision should be made by insurance against the damage to buildings caused by earthquakes in certain countries. He shows that, since the beginning of the seventeenth century, less than forty earthquakes have been responsible for deaths of more than 150,000 persons in Italy alone. Moreover, to take but one example, the great loss of life during the Ischian earthquake of 1883 was due to the fact that the buildings had already been damaged by the earthquakes of 1828 and 1881. Dr. Baratta points out some of the conditions that must determine the amount of the premium that should be demanded by insurance societies. The most important is the degree of seismicity of the district; but this would be modified by others, such as the nature of the surface-rocks, the character of the buildings, &c. One advantage of compulsory insurance against earthquakes in a country like Italy would be that partially damaged buildings would be at once rebuilt or repaired, and this would tend to diminish the loss of life in the future.

From *Nature* 14 September 1899.

50 YEARS AGO

Social Biology and Welfare

By Sybil Neville-Rolfe...

In 1905, at the age of twenty, the widow of a naval officer announced to her relatives her intention "to study prostitution and venereal disease and try to get rid of them". She knew of no organisation or senior friend under whose tutelage she could begin. With courage and energy, Mrs. Neville-Rolfe plunged into the obscurity and obscurantism surrounding the problems of sex irregularity; learning the hard way, but backed by the irrepressible gifts of her personality, she has given the last forty-five years to by no means unsuccessful efforts to induce the public and officials, both at home and abroad, to face squarely and openly some of the biological and social factors involved in healthy and unhealthy sex and family relationships in the community. ... Unfortunately, the book itself is not a useful contribution to the campaign for a rational, humane and continuously constructive approach to the problems of social hygiene, the complexities of which grow the more we know about the field. ... Mrs Neville-Rolfe's gifts are more those of a crusader than of a writer.

From *Nature* 17 September 1949.

Antagonists on the left flank

Tim King and Nigel A. Brown

The early development of vertebrate embryos is, essentially, a sequence of switches in cell fate. These switches are triggered by signals that cells receive from their neighbours. We used to think of these signals as operating directly and in isolation. Then, some signals were discovered to be antagonists, which block other signals either by taking their place at the receptor or by binding to the signals themselves. Now, several studies of left–right asymmetry in embryos — including a paper by Esteban *et al.*¹ on page 243 of this issue — beautifully illustrate how direct and antagonistic signals can operate in concert.

The ability to distinguish left from right is needed to shape and position the heart and visceral organs properly. One might imagine that the only requirement is a signal present on one side of the body and not on the other. But the reality is much more interesting. The critical signalling pathway involved uses serine/threonine kinase receptors, which bind secreted proteins of the transforming growth factor (TGF)- β family. In all vertebrates, expression of one such protein on the left flank of the embryo (the lateral plate) seems to be critical for determining 'leftness'. This protein, known as Nodal, probably does this by inducing the production of a transcription factor called Pitx2. But how is Nodal itself switched on?

In the chick (Fig. 1), the sequence of signals starts with a protein called Activin (another TGF- β ligand) on the right of the earliest

embryonic structure, the primitive streak, then runs through a region called the node, at the head end of the streak. Here, two other classes of pathway come into play. On the right, expression of the fibroblast growth factor (FGF)-8 is upregulated, whereas expression of Sonic hedgehog (Shh) is restricted to the left. We knew that Shh is needed to establish left-sided expression of Nodal in the chick². Now, Esteban *et al.*¹, as well as Zhu *et al.*³ (reporting in *Current Biology*) and Yokouchi *et al.*⁴ (in *Cell*), show us how.

The key discovery is a gene related to Cerberus (a secreted protein best known in head development). Cerberus, which is induced by Nodal, acts as a broad antagonist. As well as inhibiting Nodal itself and other members of the TGF- β family, Cerberus inhibits a second class of signals known as the Wnts⁵. Is this the case on the left or the right side of the embryo? The newly discovered gene, called Caronte, is first expressed to the left of the node. A small patch of Nodal is also expressed nearby, but this does not seem to induce Caronte. Instead, Caronte is induced by Shh and repressed by FGF-8. Caronte expression then extends to the left lateral plate, where it induces expression of Nodal by antagonizing a sub-class of the TGF- β family, the bone morphogenetic proteins (BMPs). These proteins are bilaterally expressed and they act, in the absence of Caronte, to repress the expression of Nodal. So, Caronte blocks one member of the TGF- β family — a BMP — and the result is the

induction of another member of this family, Nodal. But Caronte, like Cerberus, can also bind Nodal, suggesting a self-regulating loop — which is not without precedent, but only half the story.

Lefty-1 and Lefty-2 also belong to the TGF- β family and were first found in the mouse. Expression of Lefty-2 mimics that of Nodal in the left lateral plate, and Lefty-1 is expressed in midline structures⁶. Esteban *et al.*¹ also describe how expression of chick Lefty-1 is similar to that of Shh in the left node, and how it spreads to the left of the notochord (the midline tube that runs the length of the embryo). No doubt, expression of a chick Lefty-2 in the left lateral plate will be found in due course. Yokouchi *et al.*⁴ show that Caronte induces the midline expression of Lefty-1, again by antagonizing BMPs. Like Caronte, the Lefty proteins have wide-ranging antagonistic properties, blocking Activin⁷, BMPs⁶ and Nodal⁸. Furthermore, Esteban *et al.* suggest that Lefty and Caronte might interact directly. They base this idea on the fact that ectopic Lefty can inhibit Caronte-mediated induction of Nodal through inhibition of BMPs. So, Lefty may be able to antagonize both the ligands and their antagonist. The combinations of possible interactions are bewildering.

Perhaps Lefty-1 — induced by Shh in the left of the node — acts to block any stray Activin signals from the right. In the midline it may then prevent Caronte from spreading to the right, providing a barrier role for the midline. In the lateral plate, Caronte is responsible for inducing Nodal (and, perhaps, Lefty-2) by blocking the inhibitory effect of BMPs. Nodal then induces Pitx2, which directs subsequent morphological decisions. Although Lefty-2 could enhance the upregulation of Nodal by blocking BMPs, it could also curtail Caronte signalling or even block Nodal signalling itself. And, as already mentioned, Caronte itself may also block Nodal.

Such a delicate balance now seems to be common in controlling developmental decisions, and many classes of specific antagonist are being identified for all the signalling pathways. Why should this be? Signals often act in negative-feedback loops — not only at the level of the secreted proteins themselves, but also at the levels of signal transduction and transcriptional regulation⁹. In other words, induced factors inhibit their inducers. This allows the embryo to generate local bursts of signalling activity that are then quickly inactivated. Differential diffusion of a ligand and its antagonist can elaborate patterns or sharpen gradients. In the case of left–right determination, the need to prevent signals from crossing the midline requires such interactions, and perhaps different organs are regulated by discrete regions of the lateral plate that have disparate signal interactions. For example, there must be a region that con-

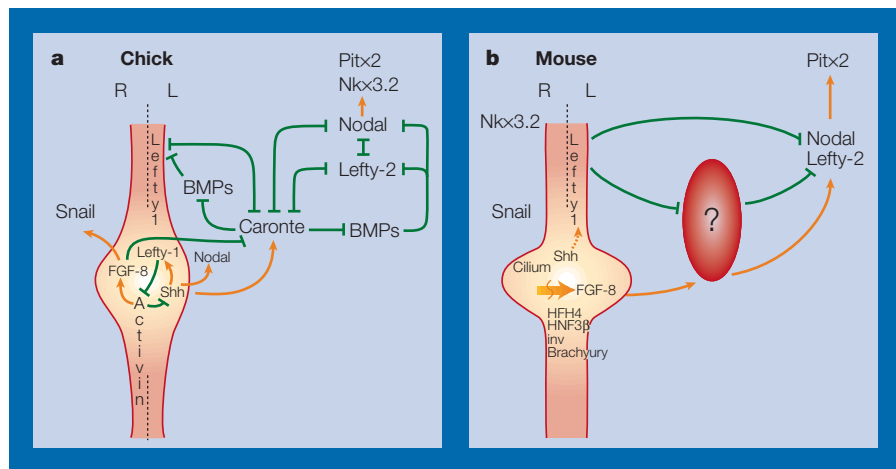


Figure 1 Signalling pathways in left–right determination. **a**, In the chick, expression of Activin upregulates expression of the fibroblast growth factor (FGF)-8 and downregulates expression of Sonic hedgehog (Shh) to the left. Shh, which is needed to establish left-sided expression of another protein, Nodal, also normally stimulates expression of Caronte. Caronte establishes the left-sided expression of Nodal by antagonizing bone morphogenetic proteins (BMPs). **b**, In the mouse, FGF-8 is required for left-sided expression of Nodal. Mutations in inversion (inv), the transcription factors HNF3 β , Brachyury and HFH4, and microtubule-associated proteins disrupt these pathways.

trols rightward looping of the heart and is not affected by Pitx2 (see the reports by Lu *et al.*¹⁰ and Lin *et al.*¹¹ on pages 276 and 279 of this issue). In the end, then, the exact timing of expression, rate of diffusion and relative thresholds of activity of all these ligands and antagonists must combine to determine patterns of cell fate.

Investigating these interactions is quite enough to be going on with, but researchers will also be keen to isolate Caronte in the mouse. Surprising differences have already come to light between the mouse and the chick (Fig. 1), including the roles of Shh^{12,13} and cilia on node cells¹⁴. In the mouse, FGF-8 is needed for left-sided expression of Nodal¹⁵, whereas it is involved on the right in the chick. Conversely, Esteban *et al.*¹ describe a second transcription factor, Nkx3.2, which is induced by Nodal on the left in the chick, but appears on the right in the mouse¹⁵. Is it a

paradox that the final asymmetries in body plans are very well conserved across species, yet their developmental pathways are not? ■

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Atmospheric chemistry

Clouds and climate

Henning Rodhe

Soapy components of air pollutants lower the surface tension of tiny, wetted, aerosol particles. This could make the particles more likely to turn into cloud drops, through condensation of water vapour on their surface, and lead to a larger number of cloud drops of smaller size. The upshot — the clouds become more efficient reflectors of sunlight and thereby tend to cool the Earth's surface. This chain of events has been investigated by Facchini *et al.* (page 257 of this issue¹), who have applied a novel empirical approach to one of the links.

Since the late nineteenth century, it has been known that clouds consist of small water droplets (or ice crystals) and that the condensation of water vapour to form such droplets takes place on pre-existing aerosol particles called cloud condensation nuclei (CCN). The conventional wisdom for many years has been that there are always enough CCN in the air — from soil dust, sea spray and other natural sources — to allow clouds to form whenever the cooling of ascending air parcels by adiabatic expansion creates supersaturation. Additional man-made sources were not believed to have any effect. Today we know that this picture is too simple. Both the physical characteristics^{2,3} and amount of cloud⁴ can be affected by man-made changes in the CCN population.

In 1921 the Swedish meteorologist Hilding Köhler formulated the now classic theory of how CCN can be 'activated' and grow by condensation to cloud droplets⁵. According to Köhler's equation, supersaturation has to exceed a critical value, S_c , not much above

100% saturation, before a droplet starts to grow spontaneously to cloud-droplet size. It turns out that S_c is a function of, among other things, the concentration of solute in the wetted aerosol particle and its surface tension. By adding soluble material to the atmosphere (for example sulphate particles derived from industrial emissions of SO₂) we are now increasing the number and the efficiency of CCN, and thereby increasing the concentration of droplets in the clouds of polluted regions². As a result, the clouds become brighter — because of an increased surface area per volume of water — and may also be longer lived.

This particular route of human influence



Figure 1 Enigmatic clouds — stratus, seen over Monterey Bay, California. In the work discussed here, Facchini *et al.*¹ estimate the extent to which organic pollutants can increase surface tension in cloud droplets, leading to an increase in droplet number and thereby to a higher level of scattering of incoming solar radiation. The significance of the effect on a global scale will, however, need further evaluation — clouds are complicated subjects of study.

on clouds and climate has been discussed at length during the past few years as a possible masking effect on greenhouse warming⁶. But because of the complexity of the processes involved and the difficulty of validating theoretical estimates by measurements, the magnitude of this indirect aerosol effect on climate has remained uncertain. Indeed, an assessment of climate change⁶ includes the corresponding estimate of global climate forcing as a wide range (0 to -1.5 W m^{-2}), with no central value given. This compares with the estimate of positive forcing of about 2.4 W m^{-2} due to the increase in greenhouse gases.

What Facchini *et al.*¹ have done is to highlight another factor in the Köhler equation, surface tension, and apply an empirical approach to estimate its magnitude. Their argument is that the presence of surfactant organic material could lower the surface tension of the wetted aerosol particles so that the critical supersaturation S_c decreases and allows more particles to become activated and grow to cloud droplets. This, again, would make the clouds brighter and lead to a negative climate forcing.

Although they are not the first to point out this possibility (Li *et al.*⁷ have done so, for instance), Facchini and colleagues' estimate of the magnitude of this effect represents a notable contribution in that it is based on an actual measurement of surface tension in a sample of water collected from ambient clouds. In this way the pollutants in the water sample, notably the organic compounds, are those present in the real atmosphere rather than the selected compounds studied in previous investigations^{7,8}. On the other hand, unlike Li *et al.*⁷, Facchini and colleagues do not consider other influences of organic pollutants on S_c . For example S_c could increase due to a reduction in the molality of the solution caused by the high molecular weight of the organics.

Facchini *et al.* collected cloud water from fog (that is, clouds at ground level) in the Po Valley in Italy. They then partly evaporated the sample to simulate the larger concentrations of the organic compounds occurring in the droplets at their activation threshold. Assuming equilibrium conditions apply, which may not be perfectly true⁹, they introduced the modified value of the surface tension into Köhler's equation and derived an estimate of the resulting decrease in S_c . Using a 'back-of-the-envelope' calculation, they went on to estimate an approximate magnitude of the global climate forcing caused by the change in surface tension due to organic surfactants, assuming that these are all man-made.

The resulting figure (almost -1 W m^{-2}) must be looked on with caution, however. For one thing, as the authors acknowledge, it is likely to be an overestimate because they assume all stratus clouds to be equally affected by those man-made organic pollutants

present in the Po Valley, even clouds occurring in remote marine areas. Marine stratus clouds have turned out to be the most susceptible to this kind of impact. Furthermore, if they had included other effects of the organic pollutants, including a possible increase in S_c due to the organic material being dissolved in the droplets, the resulting climate forcing by the organic aerosol might even have turned out to be positive⁷. So at this stage it would be premature to take the authors' estimate of climate forcing at face value.

Nevertheless, their calculation does demonstrate the potential importance of the surfactant effect on cloud brightness and climate. This is another step towards a better understanding of the complex mechanisms affecting the climate system. Obviously, much more has to be learnt about the sources, occurrence and effect on clouds of these kinds of organic compounds. But

because of the complexity of the microphysical and chemical processes involved in cloud formation, we may never be able to describe them in full detail. So such microphysical studies also have to be complemented by larger-scale ('epidemiological') measurements of cloud brightness and cloud droplet radii from aircraft and satellites. ■

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Biophysics

Singular take on molecules

Magdalena Helmer

What is life? At its most basic, it is the subtle interplay of large numbers of proteins that interact through enzymatic reactions and complex signalling pathways. The description of this molecular machinery becomes more detailed by the day, yet our understanding of biological processes at the most fundamental, mechanistic level remains poor. High-resolution structural images are of great help, of course, but what are needed are functional assays that can interrogate the inner workings of complex biological machines. Such assays do exist, and as they become more sophisticated we can expect a transformation in our knowledge of single-molecule behaviour in biological systems — that, at least, was the message to emerge from a gathering of 'single-molecule biophysicists' at the first conference* dedicated to their cause.

Technical advances have made it possible to probe the strength of chemical bonds^{1,2}, the elastic and mechanical properties of molecules^{3–5}, and the conformational changes and forces that drive molecular motors at the single-molecule level^{6–9}. Advanced optical probes enable the direct tracking of cellular processes¹⁰ and ion-channel activity (E. Isacoff, Univ. California, Berkeley; P. R. Selvin, Univ. Illinois), and ever-more inventive spectroscopic methods are being used to unravel the kinetics of enzymatic^{11,12} and photobiological reactions¹³, and the intermediates and energy-transfer processes involved. The ability to discern functional

(and presumably structural) heterogeneity in the properties of biomolecules and observe individual interactions in unprecedented detail will, it is hoped, ultimately lead to a mechanism-based and more quantitative understanding of molecular biology.

Discussions at the meeting took in all of these topics¹⁴, and more — research with DNA, for instance, which physicists use to study the dynamic behaviour and elasticity of polymers¹⁵ because it is fairly large, conformationally stable, and relatively easy to image and handle. Of late, however, physicists' interest has turned to topics such as the unwinding of duplex DNA by the helicase enzyme rep, the key step preceding read-out of the genetic blueprint. Using fluorescence resonance energy transfer, the activity of a single rep helicase can be monitored (T. Ha, Stanford Univ.). As the distance between donor and acceptor fluorophores on the two strands of a duplex increases, the efficiency of the energy transfer decreases and so reveals the extent and time-course of unwinding.

Once short segments of single-stranded DNA are generated inside a dividing cell, they can be replicated by DNA polymerase (DNAP), a molecular motor that catalyses DNA synthesis and uses part of the excess free energy released to translocate along the template strand. This activity is susceptible to mechanical force: when increasing tension is applied to the template strand (Fig. 1a,b, overleaf), DNAP replication speeds up until the tension reaches a value of about 5 to 6 pN; it then slows down rapidly and finally

stalls (B. Maier, École Normale Supérieure; C. Bustamante, Univ. California, Berkeley).

This behaviour is consistent with the stretching that causes a reduction in the entropy of the template, which in turn facilitates DNAP translocation. At higher tensions, however, the enzyme has to work increasingly hard to pull the template strand together and fit it into the 'closed' conformation that allows nucleotide incorporation; replication therefore proceeds at a slower rate before stalling. Intriguingly, a further increase in applied tension gets the DNAP going again — but in the opposite direction. That is, the enzyme reversibly switches from replication to exonuclease digestion, an ability that forms the heart of its in-built 'proof-reading' mechanism (Bustamante).

The action of DNAP is an essential step in passing genetic information to daughter cells. RNA polymerase (RNAP), by contrast, initiates gene expression. As with DNAP, the activity of individual RNAP molecules is highly variable and can be stalled reversibly by applying a force of over 30 pN or so¹⁶. But a more pressing question concerning RNAP activity is how so-called 'terminator' sequences influence gene expression. Terminators stop RNAP transcription at predetermined positions on the DNA with efficiencies ranging from nearly 0% to 100%. Single-molecule experiments (Fig. 1c) show that once an enzyme pauses, it has reached its point of no return — pausing is necessary as well as sufficient to cause termination (J. Gelles, Brandeis Univ.). This leaves the questions of how accessory factors influence competition between nucleotide addition and entry into a paused state, and whether it might be possible to control this competition for medical purposes.

DNA topoisomerase II (topo II), which is responsible for untangling replicated chromosomes before they are pulled apart during cell division, has also attracted the attention of physicists and pharmaceutical companies. The enzyme cuts a DNA segment, moves the uncut segment through the gap in the strand and then re-ligates the cleaved DNA, releasing two supercoils. This activity, driven by ATP hydrolysis, has now been observed experimentally (Fig. 1d) by following the discrete 'jumps' associated with the release of individual helical twists from a supercoiled DNA strand stretched between a glass surface and a magnetic bead (T. Strick, École Normale Supérieure). The observation of topo II clamping onto thermally activated DNA cross-overs in the absence of ATP shows both that the enzymatic cycle can be dissected into its constituent parts, and that this first step is not chemically fuelled.

When applied to the right questions, single-molecule biophysics provides answers that are difficult or impossible to obtain from bulk experiments. It is now possible to look beyond the ensemble average, and to dissect

*Single Molecule Biophysics, Tours, France, 8–15 July 1999.

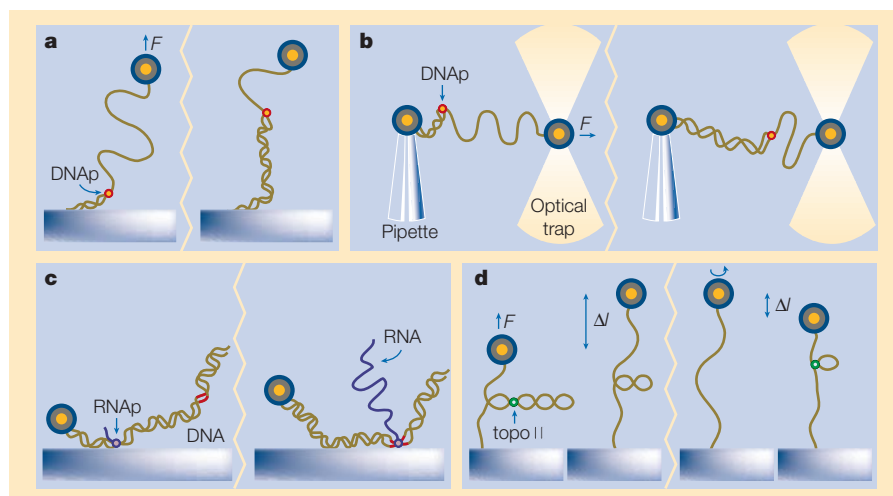


Figure 1 Single-molecule experiments. **a**, The ends of single-stranded DNA (ssDNA) are attached to a glass surface and a magnetic bead. Fluctuations in the bead's Brownian motion give a measure of the applied force F . Addition of nucleotides initiates DNA replication by DNA polymerase (DNAP) (**a**, right panel). As the elastic properties of ssDNA differ from those of double-stranded DNA, the extent of replication can be monitored by measuring changes in the extension of the DNA molecule as a function of force. **b**, Single-stranded DNA is attached to two beads held in a pipette and optical trap. DNAP synthesis of a second DNA strand is initiated by nucleotide addition (**b**, right panel) and monitored by following changes in the system's extension at a fixed applied force or its tension if the two beads are held at a fixed distance. **c**, An RNAP attached to a glass surface is 'fed' with template DNA, whose downstream end carries a polystyrene bead. The length of the DNA tether is inferred from the bead's fluctuations. The complete template is transcribed unless a terminator DNA sequence (red) induces the enzyme to pause (**c**, right panel) and stop. A lack of increase in tether length over time indicates pausing, whereas termination results in the release of the DNA template and RNA transcript. **d**, The activity of individual topoisomerase II (topoII) molecules as a function of ATP concentration can be monitored by following the discrete jumps (Δl) in the length of an ssDNA molecule (**d**, left panel). Rotation of the magnetic bead induces the formation of thermally activated DNA cross-overs, which are 'clamped' by topo II, even in the absence of ATP, thus reducing the effective length of the DNA strand by Δl (**d**, right panel).

the interaction forces and conformational changes associated with fundamental biological events. Even protein folding is being brought within the reach of single-molecule observations (X. Zhang, Stanford Univ.), through the combined use of micro-mechanical manipulation and optical probes.

No doubt new challenges will emerge to add to the existing questions. As H. Berg (Harvard Univ.) and H. Buc (Inst. Pasteur) pointed out, there are two prerequisites for tackling them successfully. First, physicists must get to grips with biology to identify the most relevant biological questions. Second, they will have to develop new physical concepts that allow for a meaningful interpretation of molecular 'individuality', the inevitable structural and functional fluctuations captured in single-molecule experiments. The trick — to paraphrase K. Kinoshita (Keio Univ.) — will be to distinguish between the fluctuations that are telling us something profound about the translation of molecular events into macroscopic behaviour, and those that should be ignored.

Magdalena Helmer is a Senior Editor at Nature.

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erratum In Daniel Weihs' News and Views article "No hibernation for basking sharks" (*Nature* **400**, 717–718; 1999), which discussed a paper by David Sims (*Proc. R. Soc. Lond. B* **266**, 1437–1443; 1999), it was stated that the energy content of copepods was previously assumed to be three orders of magnitude larger than currently accepted. This should have read three orders of magnitude smaller. Likewise, previous estimates of the energy cost of swimming should have been between one and two orders of magnitude too small — not, as stated in the article, too big. These errors make no difference to the overall conclusions discussed in the News and Views article.

Daedalus

Unchained energy

In an atomic explosion, an initial neutron collides with a nucleus in the charge. It fissions, releasing more neutrons, which fission more nuclei, and the resulting fast chain reaction runs away. But a practical bomb cannot wait for a random neutron to set it off. It fires a quick blast of neutrons into the charge at the moment of initiation. Enormously amplified by successive generations of fission, they get the chain-reaction off to a flying start.

Daedalus is now adapting the idea to the explosions in an internal-combustion engine. They too are chain reactions, the links in the chain being the free radicals liberated by combustion. Sadly, these chains are usually terminated prematurely. Unburnt and partly burnt fuel emerge in the exhaust, polluting the atmosphere.

So Daedalus's new 'radical engine' has a powerful xenon ultraviolet flash-lamp directed into each cylinder. A brief instant after the charge is ignited, the flash-lamp fires, suddenly photolysing the mixture and flooding it with radicals ripe for chain-amplification. Combustion roars away. With radical creation outpacing quenching, it goes to completion at last. Only carbon dioxide and steam emerge from the exhaust.

The radical engine will need careful optimizing. Fired too soon, a flash may detonate the whole charge very suddenly, causing engine 'knock'. Fired too late, it may shine uselessly on a few dying radical chains. Possibly several spaced flashes of different intensity will do the best job. But when properly understood, radiant radical-amplification will give internal combustion a splendid new lease of life. Not only will a radical engine be cleaner, and able to burn all sorts of fuels now too smoky or sluggish for the job. By varying the timing and intensity of its flashes under electronic control, it will be more efficient, too. It will be able to burn the charge faster or slower, optimizing the pressure-profile of each power-stroke for the current speed and load.

The technique might also work with solid high explosives. In mines, tunnels and similar confined spaces, truly clean and fumeless blasting would be very welcome. And fast-acting flash electronics might produce what is now only a figure of speech — a true controlled explosion. With its pressure adjustable in space and time, it could be a splendid tool for forming and shaping hard, intractable alloys and ceramics. Indeed, it could even lead to new and neater designs of atomic bomb.

David Jones

Up close and personal to atoms

Ali Yazdani and Charles M. Lieber

The latest microscopes provide a new level of sophistication not only in imaging but also for interacting with matter at the atomic scale.

Microscopes have been pivotal in opening new frontiers in science. The optical microscope revolutionized biology by allowing scientists to image the living cell, and the advent of electron microscopy has shaped much of our understanding of the structure of cells and inorganic materials. The invention and development of scanning probe microscopy has taken the ability to image matter to the atomic scale and opened fresh perspectives on everything from semiconductors to biomolecules. But researchers are not just passive observers; they are devising methods to modify and measure the microscopic landscape and so explore its physical, chemical and biological features.

The scanning revolution

The invention of the scanning tunnelling microscope (STM) by Binnig and Rohrer in the early 1980s — marked by the award of a Nobel prize in 1986 — was the catalyst of this technological revolution¹. In the STM, a sharp metallic tip (ideally terminating in a single atom) is positioned within a few atom-widths of a conducting surface. Such precise spatial control is achieved using piezoelectric ceramics, which change size ever so slightly in response to an electric field. When placed near the sample, electrons quantum mechanically 'tunnel' between the tip and the surface of the sample. This tunnelling process is sensitive to any overlap between the electronic wavefunctions of the tip and sample, and depends exponentially on their separation. The STM makes use of this extreme sensitivity to distance. In practice, the tip is scanned across the surface, while a feedback circuit continuously adjusts the height of the tip above the sample to keep a constant tunnelling current (typically 10^9 electrons s^{-1} , comparable to the rate of tunnelling between atoms in a metal of about 10^{13} electrons s^{-1}). The recorded trajectory of the tip creates an image that maps the electronic wavefunctions at the surface, revealing the atomic landscape in fine detail.

On semiconductor and metallic surfaces, the STM can easily resolve single atoms, and probe the structural and electronic properties of these surfaces (Fig. 1). From the outset, STM imaging has been used to resolve long-standing questions about how atoms arrange themselves differently at the surface of a material compared to the way they do in the interior. These differences are crucial to

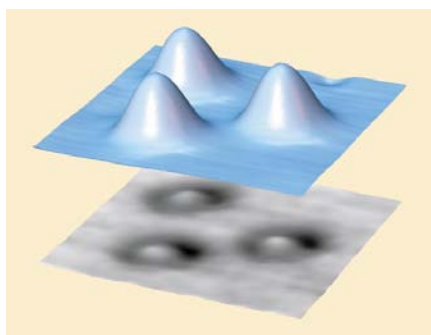


Figure 1 **The dimension beyond the images.** Spectroscopy of three magnetic atoms on the surface of a superconductor. Top, the STM topograph; bottom, an image constructed from spatially resolved spectroscopy showing dark patches where superconductivity is suppressed near the magnetic atoms⁶.

understanding technologically important processes such as surface-induced catalysis, growth of thin films and microfabrication techniques used in the semiconductor industry.

The STM works best in the stringent conditions of an ultrahigh vacuum, in which clean conducting surfaces can be prepared. Over the past decade, STM has led to other nanometre-scale imaging techniques that do not rely on vacuum tunnelling, but still map the interaction of a pointed probe with the sample. Collectively these new instruments are called scanning probe microscopes (SPMs). The most widely used SPM, which can operate in air and liquids, is the atomic force microscope (AFM), an instrument that maps the forces between the tip and the sample. In this microscope, a tip is mounted at the end of a soft cantilever that bends when the sample exerts a force on the tip. By optically monitoring the cantilever motion it is possible to detect tiny chemical, electrostatic or magnetic forces, which are only a fraction of those required to break a single chemical bond or to change the direction of magnetization of a small magnetic grain. Applications of the versatile AFM include *in vitro* imaging of biological processes².

Imaging with SPMs has brought us up close and personal to single atoms, molecules and molecular assemblies, and techniques are advancing to include more interactive experiments by using these instruments as nanoscopic tools rather than microscopes. Precise measurements of the local interactions between SPM probes

and materials — so-called spectroscopic measurements — give a new dimension of information about physical, chemical and biological processes on the nanometre scale. Such data are obscured or lost in conventional 'macroscopically averaged' measurements. The strong interaction between the probe tip and the sample has also been used to move atoms around on the surface, allowing the construction of nanostructures. Such techniques are at the heart of new explorations of physics on scales comparable to an electron's wavelength, as well as chemistry and biology on the scale of single molecules.

High-resolution spectroscopy

Binnig and Rohrer's original motivation was to develop a spatially resolved spectroscopic tool for studying surfaces¹, rather than to build a microscope. Tunnelling spectroscopy has been an important technique in solid-state physics since the 1960s, when studies of planar metal-oxide tunnel junctions helped confirm the theory of superconductivity in metals. But whereas planar tunnelling, like other spectroscopic techniques, gives only spatially averaged information, the STM can directly read spatial variation in electronic phenomena. In practice, spectra are measured with a metallic tip held at a fixed height above the sample, which monitors the tunnelling current as the voltage difference between the tip and the sample is varied. Tunnelling spectra are typically determined by the local density of electronic states in the sample and can be used to examine their energy and spatial dependence.

High-resolution spectroscopy was a goal of STM from the start, but its progress was limited because the requirements for vibrational stability and low electrical noise are far higher than for imaging. In fact, the first success with STM spectroscopy was in studying the voltage- and current-dependence of STM images of semiconductor surfaces^{3,4}. Since then, the energy resolution of STM spectroscopy at low temperatures has reached microelectronvolts — comparable to the best spatially averaged techniques. And as Binnig and Rohrer hoped, high-resolution spectroscopy is possible over length scales of a few tenths of a nanometre. Now STM is being used to investigate spatial variation in electronic phenomena, such as superconductivity and magnetism, which up to now have been mostly studied using macroscopically averaged techniques.

One of the first uses of high-resolution STM spectroscopy at low temperatures was to image the individual magnetic vortices threading a superconductor⁵. The instrument was used to map electronic states near isolated vortices and to show unpaired electrons bound to their cores (superconducting electrons usually travel in pairs). This work inspired further studies of superconductivity near isolated defects, as well as studies of the competition between magnetism and superconductivity on the atomic scale, where magnetic impurities can induce localized, unpaired electronic states analogous to those found at vortex cores⁶ (Fig. 1). Similar STM experiments have been carried out on metallic surfaces to examine the physics of electron scattering from magnetic atoms. Such studies detected the screening of magnetic atoms by conduction electrons — the so-called Kondo effect, which dominates the conductivity of metals doped with magnetic impurities at low temperatures^{7,8}. Advances in the use of ferromagnetic tips (such as iron) have led to spin-selective spectroscopy, which will make it possible for magnetism to be probed even more closely⁹. Such high-precision studies are, in turn, driving new theories about the local behaviour of electronic states and the spatial variation of electronic phenomena — previously thought impossible to measure directly.

Probing nanostructures

In the realm of the very small, nothing beats the STM for studying nanometre structures and the electrons confined within them. Work on semiconductors has established the

importance of electron energy levels and conductance quantization for future miniaturization of electronics. Imaging and spectroscopy with the STM are powerful ways to study these electronic effects in the next generation of small structures. One example, and an icon of STM experiments, is the quantum corral¹⁰. Here, assemblies of atoms are used to confine surface electrons, so that they display wave-interference phenomena and occupy quantized energy levels. Similar phenomena occur in chemically fabricated nanostructures, such as carbon nanotubes or semiconductor nanocrystals, and STM provides a new way to study them. For example, in isolated nanotubes, STM experiments have been used to probe the one-dimensional electronic states of these tubes, which may one day act as 'quantum wires'^{11,12} (Fig. 2). In even smaller structures, researchers are using STM as a local electrode to investigate the effects of adding electrons to these structures and to extend our understanding of charging effects and conductance quantization at the single-molecule and atom limit^{13,14}. At these extreme limits, tunnelling spectroscopy is also being used to examine the electronic and vibrational states of individual atoms and molecules at surfaces (Box 1).

Structures made up of just a few atoms or molecules have quantized energy levels, through which electrons can move, and these may one day form the basis of nanometre-sized electronic devices. The STM will be vital in this work — for example, STM studies of carbon nanotubes have linked the structure of the tubes with their conducting

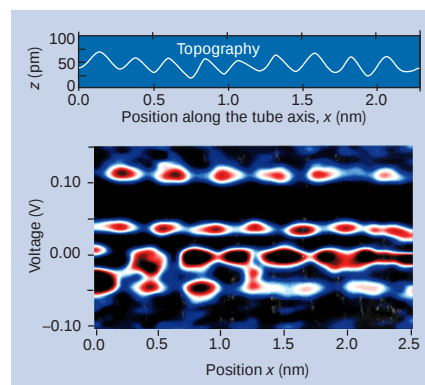


Figure 2 In carbon nanotubes, STM images both the atomic corrugation (topography, top) and the quantized electronic standing waves (spectroscopy, bottom). The periodicity in the spectroscopic image is determined from the square amplitude of the electrons' wavefunctions at discrete energies (voltage)³⁵.

or insulating behaviour^{11,12}. Such data are almost impossible to obtain from a macroscopic measurement of a collection of nanotubes, because nanotube fabrication naturally yields a distribution of tubes with different structures, sizes and defect densities.

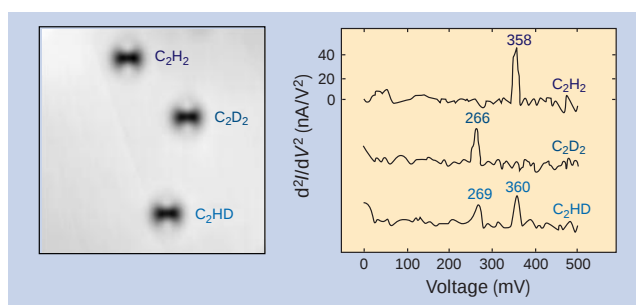
Forces in chemistry and biology

The size and range of intermolecular forces determines many critical processes in biology and chemistry, from general membrane assembly to specific binding and recognition. One of the most powerful tools for studying these processes is AFM, which has a unique ability to measure small forces with

Box 1: Fingerprinting individual molecules

The atoms in a molecule vibrate against each other at characteristic frequencies determined by the strength of their chemical bonds. Different vibration modes and their frequencies make up a 'fingerprint', which identifies molecules and pinpoints changes in their bonding owing to chemical reactions. For example, when a molecule is absorbed at a surface, depending on the location or orientation of its absorption site, its vibrational modes may shift in frequency or be suppressed. Knowing these changes is critical to understanding a variety of surface phenomena, such as absorption and release processes, heterogeneous catalysis and epitaxial growth.

Since its invention, the scanning tunnelling microscope (STM) has had the potential to perform site-selective measurements of



vibrational spectra of single molecules³². This approach was motivated by the success of much earlier experiments in planar metal-oxide tunnel junctions, when the tunnelling conductance was shown to make characteristic jumps at energies corresponding to the characteristic vibrational frequencies of molecules adsorbed at the metal-oxide interfaces in these devices. The underlying principle is that when

the energy of the tunnelling electron exceeds that required for exciting a molecular vibrational mode, electrons can tunnel inelastically as well as tunnelling between states of the same energy. This means that they lose energy by causing a molecule near the tunnel junction to vibrate. Sharp steps in the measured conductance at different energies correspond to the onset of inelastic tunnelling processes and provide the

fingerprint of the molecular vibrational modes.

A group at Cornell University, using an STM operating at low temperatures and in ultrahigh vacuum, has succeeded in reproducibly measuring the vibrational spectra of individual molecules²⁸. Most impressively, they have used inelastic tunnelling spectroscopy to identify individual molecules by their characteristic vibrational energies³³. The STM image and inelastic spectra of three similar molecules, C₂H₂, C₂D₂ and C₂HD are shown here. Although STM imaging cannot distinguish between the three molecules, the inelastic tunnelling spectra measured with the tip can reveal their vibrational fingerprints and identify them. The peaks correspond to incremental increases of conductance associated with exciting the C–H and C–D bonds.

A. Y.

high spatial resolution. Usually, AFM is used to record the surface topography of a sample by recording the vertical motion of the probe tip as it is scanned over the sample. With a customized probe tip, however, specific interactions between the tip and sample surface can be measured. For example, the self-assembly of organic monolayers on AFM tips has been used to explore the surfaces of organic and polymer materials¹⁵. Such 'chemical force microscopy' can record differences in the intermolecular forces between the tip and different sample regions, and thereby map chemically distinct regions. Spectroscopy can potentially provide a new dimension of information (Box 2), and, with AFM, it is force spectroscopy — the measurement of force against separation — that researchers have been developing. Force spectroscopy with customized probes can measure how strongly things stick together, which is essential if you are planning to build nanostructures.

The way things stick together is especially critical in biology, defining, for example, ligand–receptor interactions and protein folding. Force spectroscopy can probe these processes at the single-molecule level. In a typical experiment, the ligand is attached to an AFM tip, receptors are linked to the substrate, and then the two are brought together and separated again. To be sure that only a single ligand–receptor interaction is measured, the active species are attached at a low density to the tip (which is much bigger than most biological molecules). Notably, measurements of force against separation for the protein receptor avidin and its ligand biotin have shown a clear signature for the unbinding of single biotin molecules from the protein, demonstrating that single ligand–receptor events can be investigated¹⁶ (Fig. 3, overleaf). This approach, or simply adsorbing much larger molecules onto AFM probes, has been used in force spectroscopy experiments to investigate the unfolding of multidomain proteins and structural changes of carbohydrate polymers^{17–19}.

One of the attractions of these single-molecule force spectroscopy experiments is that they allow a quantitative dialogue with theory that benefits both¹⁹. But these experiments have their limits and there is competition from other techniques. For example, optical tweezers, which work by trapping micrometre-sized beads at the focal point of a laser beam, have considerably better force resolution than AFM, although they must be used with biological structures larger than the beads. The AFM should excel in the investigation of individual proteins, polymers and submicrometre assemblies, and such studies could be particularly fruitful because microscopic changes, for example of single nucleotides or amino acids, can be made using standard chemical and biological techniques. But it will be necessary to

define exactly where an 'active' ligand is attached relative to the probe surface, something that has not been possible using conventional AFM tips. Tips made from carbon nanotubes — modified to measure single ligand–receptor unbinding — can overcome this limitation, and may help us image biological interactions with molecular precision²⁰.

Making atoms move

Almost 40 years ago, Richard Feynman²¹, speculated that "in the great future — we can arrange the atoms the way we want; the very atoms, all the way down!". Now SPMs, with their capacity to cause controllable changes on the nanometre scale, give us this power^{22,23}. The idea behind SPM lithography is to exploit the forces between probe and

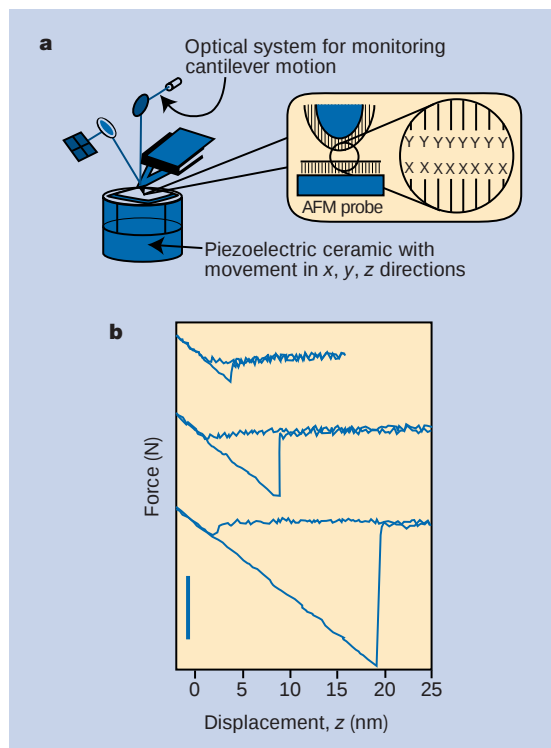
sample (which are always present in these microscopes but usually kept to a minimum). For example, during imaging, the tip of an STM is held sufficiently high above the sample to reduce the chemical interaction between probe and sample. When it is much closer, the tip can exert enough force to move individual atoms or molecules and to modify the atomic landscape. The interaction between the tip and atoms or molecules bound to the surface (adsorbates) can be repulsive or attractive, allowing the tip to push or pull individual adsorbates on the surface. These techniques have been used to construct impressive nanostructures, such as the 'quantum corral' (by moving single atoms)¹⁰ and the 'molecular abacus' (by moving individual C₆₀ molecules)²⁴.

The SPM can also be used to modify a

Box 2: Fingerprinting chemical bonds

In atomic force microscopy (AFM), molecular groups, such as carboxylic acids (–COOH) can be added to the tip. Separating the tip and sample deflects the cantilever–tip assembly until the restoring force exceeds the bonding interaction — at this point there is a mechanical instability and the tip and sample jump apart. The magnitude of the cantilever deflection immediately before the onset of this instability can be used to calculate the intermolecular bonding interaction: $F_{\text{bind}} = k_{\text{cant}}\Delta x$, where F_{bind} is the binding force, k_{cant} is the spring constant of the cantilever–tip assembly and Δx is the displacement. This approach has been used to distinguish different types of non-covalent bonding¹⁵ (shown here) and different ligand–receptor interactions, and to investigate the breaking of single covalent Si–C bonds³⁴.

The figure shows a customized tip (a) and force spectroscopy data (b) that can distinguish between different bonding interactions. In a, a tip modified with a monolayer terminating in the functional group Y is shown probing a sample that terminates in the functional group X (where X and Y can be either COOH or CH₃ groups). Force spectroscopy data in b show clear differences in breaking COOH–COOH, CH₃–CH₃ and



COOH–CH₃ non-covalent bonds.

Such experiments are technically impressive and promise to expose the details of interactions at the single-bond level. But some thorny issues have to be addressed first. For example, the detailed orientation of the CH₃–CH₃ bond in the above experiment, which defines the reaction coordinate — that is, the direction along the potential energy surface describing the interaction

between the groups — was not known because of the large curvature of the tip and other modifications. Knowledge of the reaction coordinate is necessary for a detailed and meaningful comparison of experiment with theory. A solution to this vexing problem may come from customized carbon-nanotube tips, which are now enabling researchers to localize single molecules in a precisely defined orientation at the end of a molecular-sized probe²⁰. **C. M. L.**

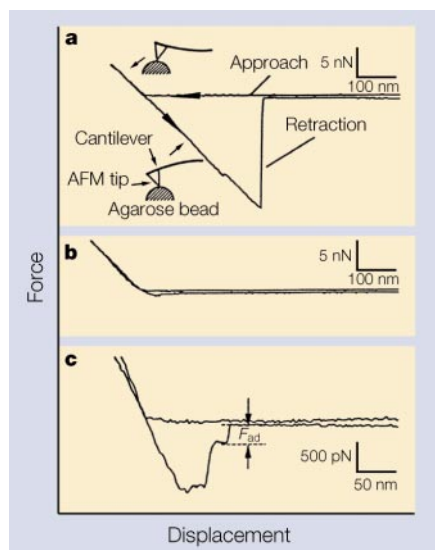


Figure 3 Biological forces. The adhesion force between an AFM tip terminating in avidin and agarose beads coated with biotin is measured by recording the deflection of the AFM cantilever on approaching and retracting from the bead¹⁶. The strong interaction recorded in a can be blocked by adding an excess of free avidin to the solution (b). When the biotin is only partially blocked by avidin, unbinding occurs in discrete steps (c), corresponding to an adhesion force, F_{ad} , of 160 ± 20 pN.

sample indirectly by applying large electric fields or injecting energetic electrons^{22,23}. An intense local electric field can change the energy of chemical bonds at the sample surface and reduce the barriers that hinder the motion of atoms and molecules. Local injection of energetic electrons can be used to excite the electronic or vibrational modes of molecules, which can result in breaking of individual bonds. In this way, STM experiments have demonstrated translation²², rotation²⁵, desorption²⁶ (or release of adsorbates) and dissociation²⁷ of individual molecules at the surface.

Quantitative measurements of the interaction of SPM probes with adsorbates and surfaces provide new insights into the physical mechanisms of molecular motion. A step towards detailed understanding of STM-induced modifications has been the development of inelastic tunnelling spectroscopy with the STM²⁸ (Box 1). This is a quantitative way of directly measuring the frequency of vibrational modes of individual adsorbates, providing a link between vibrational excitation and atomic motion on the surface.

Precision lithography

Progress in exploring SPM-induced modification of materials points to an exciting future for SPMs as an alternative high-precision lithographic tool for making nanometre-sized electronic devices. Functional devices, such as single-electron transistors, have already been created²⁹ using the STM and AFM to apply intense electric fields to conducting films, oxidizing them in a pattern that defines the device structures. Of course, plenty of hurdles will have to be overcome to compete with conventional methods of lithography, which can create three million transistors in a single chip. One problem is the serial nature of SPM lithography, which is associated with relatively slow throughput, but it can be addressed using arrays of SPM probes to perform imaging and lithography simultaneously (Fig. 4)³⁰. Work is underway to make even larger arrays, perhaps with ten thousand probes; the engineering challenge lies in developing circuits for controlling the probes in parallel and for real-time processing of the large-scale images they produce.

Another problem with using SPMs for lithography is the task of making probes that can withstand the forces exerted on them during the lithographic process. These forces can lead to uncontrolled modification of the AFM or STM tips, making them unreliable tools. Fortunately, carbon nanotubes appear to have all the properties required of an ideal tip; they are chemically inert, mechanically resilient and can be electrically conducting. Such tubes have been used as robust tips for both imaging and lithography³¹.

Continuing evolution

The SPM has evolved from a passive imaging tool into a sophisticated probe of the nanometre scale. These advances point to exciting opportunities in many areas of physics and biology, where SPMs can complement macroscopically averaged measurement techniques and enable more direct investigations. More importantly, these tools should inspire new approaches to experiments in which controlled measurements of individual molecules, molecular assemblies and nanostructures are possible. The future will also be shaped by the relevance of these probes to technology, such as their possible use as electronic or magnetic memory devices or for advanced

lithography. Indeed, the rapidly growing number of versatile scanning probes, besides the STM and AFM discussed here, although remaining primarily research tools, will strongly influence the development of nanometre-sized electronic devices and biological sensors.

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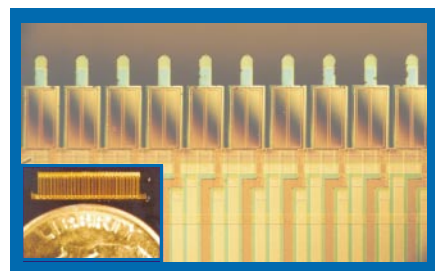


Figure 4 Thousands of tiny tools. A 1-cm linear array of 50 high-speed scanning probes with integrated sensors and actuators fabricated using micro-machining techniques. The spacing between the tips is 200 μm . The inset shows the entire array next to a US dime, whereas the full image shows a magnified view of ten probes.

Food contamination by PCBs and dioxins

An isolated episode in Belgium is unlikely to have affected public health.

In February 1999, a poisoning episode broke out in several poultry farms in Belgium. The Belgian authorities took immediate safeguards to protect public health and implemented a large-scale food-monitoring programme. Here we analyse the scale of the contamination and assess the likelihood of its adversely affecting the health of the general population.

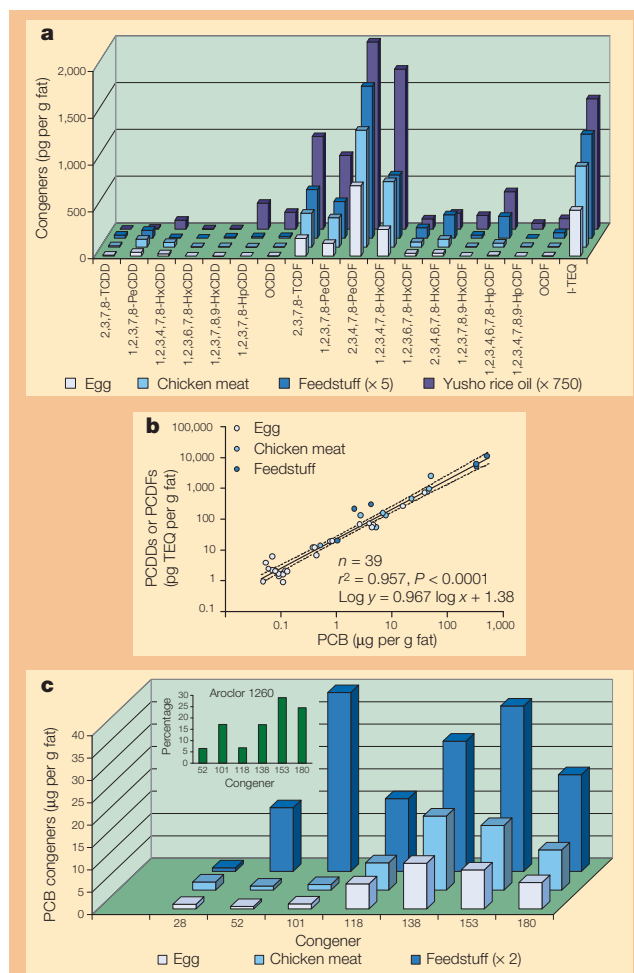
The first symptom of contamination was a sudden drop in egg production. A few weeks later, there was a marked reduction in egg hatchability, along with reduced weight gain and increased mortality of chicks. These birds presented with ascites, subcutaneous oedema of the neck and neurological disturbances (ataxia). Histology revealed degenerative changes of the skeletal and cardiac muscles. The lesions resembled 'chick-oedema disease', which was reported during the 1950s to 1970s in several outbreaks of poultry poisoned by polychlorinated biphenyls (PCBs) and dioxins¹, suggesting that dioxins might be responsible for the Belgian cases. High concentrations of dioxins in chicken meat and feedstuff were subsequently found and, at the end of April, the source of dioxins was traced to a stock of recycled fat that had been delivered to a manufacturer of animal feed in mid-January.

Analysis of contaminated feedstuff and poultry products showed a consistent pattern of dioxin congeners, dominated by the polychlorodibenzofurans (PCDFs) (Fig. 1a). Polychlorodibenzodioxins (PCDDs), in particular the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin involved in the 1976 Seveso accident in Italy and in contamination by the Agent Orange defoliant, were present only in minor proportions (less than 5%) of total congeners. This pattern of PCDD and PCDF congeners was virtually identical to that in the contaminated rice oil that poisoned 2,000 people in 1968 in Yusho, Japan².

All contaminated samples contained increased amounts of PCBs, which closely correlated with the concentrations of PCDDs and PCDFs (Fig. 1b), having an average ratio of PCB:PCDD/F of about 22,000:1. The PCB pattern in feedstuff was closely matched to a PCB mixture composed mainly of Aroclor 1260 (Fig. 1c). In contaminated poultry meat and eggs, the distribution of PCB congeners was altered because of the preferential biological transformation and clearance of the lower chlorinated congeners (PCB 52 and 101). These findings led us to use the PCB analysis test to identify contaminated food products.

By using this test to survey PCB contamination of food, we confirmed that it was

Figure 1 Dioxin and PCB congeners in contaminated Belgian poultry. **a**, Comparison of 2,3,7,8-substituted PCDD and PCDF congeners between Yusho rice oil and contaminated poultry. Each value is the mean of two (feedstuff and chicken) or five samples; egg samples are a mixture of 10 to 12 eggs. Total dioxin activity was calculated by using the 1998 international toxic equivalent factors (I-TEQ) of the World Health Organization. Values for feedstuff and Yusho oil have been divided by 5 and 750, respectively, for ease of representation. **b**, Correlation between PCDDs or PCDFs and PCBs in poultry feedstuff, chicken meat and eggs. PCB values correspond to the sum of the seven mono- and multi-*o*-PCB marker congeners (IUPAC 28, 52, 101, 118, 138, 153, 180) measured by high-resolution gas chromatography/mass spectrometry. **c**, Distribution of PCB congeners in contaminated poultry feedstuff, chicken meat and eggs (samples as in **a**). Values for feedstuff have been divided by 2.



mainly poultry that was affected, with PCB concentrations in eggs and chicken meat being up to 250 times the tolerance level of 0.2 µg per g fat (Fig. 1b). Some pigs were also contaminated, but to a lesser extent than chickens, with up to 75 times the tolerance level, and no symptoms of poisoning were observed. In contrast, bovine livestock were free of contamination: from a total of 1,320 milk samples, only two were slightly (40 and 60%) above the tolerance level for PCBs (0.1 µg per g fat) but were within the tolerance level for dioxins (5 pg I-TEQ per g fat).

These findings can be explained by the longer life cycles of pigs and cattle and their lesser dependence on manufactured food mixtures compared with chickens. They also reflect the total amount of PCBs and dioxins introduced into the animal feed. By extrapolating the concentrations in chicken feedstuff to the initial volume of contaminated fat (maximum, 80 tonnes), the total amount of dioxins inadvertently mixed in the recycled fat can be estimated at about 1 g and that of PCBs at about 50 kg. Theoretically,

such quantities could contaminate millions of chickens but have no effect on other livestock on the 3,000 farms that may have taken delivery of contaminated feed.

The outbreak of dioxin contamination in Belgium is an almost exact replica of the outbreaks of poultry poisoning by polychlorinated compounds that occurred repeatedly in the United States and Japan in the 1950s to 1970s. These outbreaks produced the same effects on the avian reproductive system as the Belgian contamination. Two of these previous reports also involved PCB mixtures (Kanechlor 400 and Aroclor 1242)^{3,4}.

As in earlier accidents, it is very unlikely that the isolated episode of contamination in Belgium will cause adverse health effects on the general population. It would require the consumption of 30–40 meals of highly contaminated chicken or eggs (dioxin levels of around 1,000 pg TEQ per g fat) to double the body's PCB and dioxin burden. Even in such an extreme case, the maximum body burden would still be at least a factor of

one hundred less than in the victims of the Yusho accident and in the Seveso residents (referring to the mean value in the most contaminated zone), but would be similar to the two- to threefold increase in PCB and dioxin body burden of subjects affected in the 1980s⁵ or of those regularly eating contaminated seafood⁶.

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Reproductive biology

Pheromones and regulation of ovulation

There is controversy surrounding the issue of whether there is menstrual synchrony in women who live together^{1–4}, particularly in the case of the coupled-oscillator model developed⁵ to explain similar data from rats. Stern and McClintock⁶ have proposed that the rat model applies to women, with the effect being mediated by two opposing axillary ‘pheromones’ that could affect major reproductive events and have potential for “either contraception or treatment of infertility”.

This claim⁶ is based on data derived from four cycles from each of 20 subjects treated with axillary compounds to change the cycle length from that of each subject’s baseline cycle. Subjects’ upper lips were wiped daily with pads worn in the axillae of donors in follicular or ovulatory phases of their cycles. The cycles of subjects wiped with follicular pads appeared to be shortened by 1–14 days, whereas cycles of those wiped with ovulatory pads were longer by 1–12 days. One-third of cycles did not change or changed in the opposite direction.

My main criticism of the study is the use of the value of the single first cycles, receiving carrier-only treatment, to derive the data analysed. Such single observations have no within-subject variance and the irregular statistical manoeuvre of converting all 20 observations to zero masks any between-subject variance and provides an illusory zero baseline with indeterminate confidence limits. Carrier-only treatments should have been distributed throughout this long experiment to give a balanced crossover design with three treatments (carrier, follicular and ovulatory) and two or more complete replications to confer confidence limits to the baseline observations, thus making comparisons valid.

Each group has an apparent outlier favourable to the model: one of –14 comprises 25% of the total shortening, whereas

that of +12 makes up 22% of the increase. Excluding these two outliers would abolish the claim of significance.

Reference to the first cycle throughout the long experiment may have introduced biases because of environmental, physiological and social drifts. McClintock¹ reported that women “who estimated seeing males less than three times per week” had cycles 1.5 days shorter than those who spent more time with males. Others claim that cycles are affected by sexual activity or the phase of the moon^{7,8}.

I am not convinced of the validity of the coupled-oscillator model derived from rat studies⁵. I also question the “definitive evidence” that pheromones regulate human ovarian function because, if these exist, their characterization will require large, carefully designed experiments, a controlled social and physical environment, and a clearly defined endpoint measured in hours.

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McClintock replies — We first reported menstrual synchrony almost three decades ago, and it has since been verified repeatedly. But synchrony does not always occur, and the circumstances in which it does not occur tell us a great deal about the social and physical conditions required^{1,2}. Menstrual synchrony is but one manifestation of a more fundamental mechanism, the chemosensory regulation of ovulation, which occurs in other species not only as synchrony, but also as asynchrony, extreme cycle regularity, changes in the timing of puberty, birth cycles and reproductive ageing^{1,2}.

One of the studies³ cited by Whitten to support his statement that synchrony is still controversial describes a non-random relationship between the cycles of lesbians living together, albeit not in synchrony. It is therefore consistent with our idea that ovarian-modulating chemosignals provide a basic mechanism that also gives rise to diverse forms of social regulation of ovulation.

Whitten implies that our data were poorly controlled, skewed and selected, but his criticisms do not hold with a careful reading of the experimental details⁴. The main criticism is based on a misreading of our procedure for within-experimental-subjects control. Each woman in the group was studied for five, not four, cycles and was exposed first to the carrier and then to follicular and ovulatory axillary compounds (in balanced randomly assigned order). We did not ignore the environmental, physiological and social factors that cause variation between women in the length of the menstrual cycle to be greater than the variation within women. Our within-subjects design controlled for this, and included the standard normalization procedure of expressing data as a change in cycle length from the baseline cycle preceding each condition. Statistical analyses were done only on these four change-scores (the baseline cycle lengths, all standardized to zero, were only depicted graphically).

Two other analyses also demonstrated the effect of human axillary compounds on ovarian function. A second control group of women who received only the carrier (frozen and thawed undercast pads with a few drops of alcohol) were significantly different from the experimental group, who also received both types of axillary compound.

Third, we analysed the absolute length of cycle phases, not change scores, by using log-survivor analysis developed for temporal data⁵. All temporal data are “skewed” and randomly have a Poisson, not a normal, distribution, and extreme values are not “outliers” but part of the temporal distribution. Survivor analysis indicated that axillary compounds change the timing of the preovulatory surge of luteinizing hormone, rather than the onset or duration of menses or the lifespan of the corpus luteum. So we did use a “clearly defined endpoint measured in hours”: the timing of the preovulatory surge of luteinizing hormone⁶, and not just the coarser measure of menstrual-cycle length. The concordance of our three independent statistical methods creates a robust foundation for concluding that axillary compounds significantly change the length of the menstrual cycle by altering the follicular phase.

Our log-survivor plot confirmed that the main effect is not driven just by the extreme cases, and their removal does not “negate the claim”. Moreover, the extreme

cases do not contribute as much to the effect as Whitten speculates. We emphasized variation in response so future investigators would not expect all subjects to have the mean response. Our computer model⁷ showed that such individual differences are necessary for the patterns we see in nature. Determining the basis for these differences is an important topic for future research.

Our comparative approach and use of computer modelling do not imply that rats and humans are the same, or the same as computers. They merely suggest that the various forms of social regulation of ovulation in these species can be predicted by conceptualizing their interactions as a system of mutually entrained oscillators. Our corroborated model enabled us to broaden our original focus on synchrony, which is only one special case, to fundamental phero-monol signals that can change the time of ovulation in various ways.

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Enterobacteria

Antibiotic resistance found in wild rodents

Resistance to antibiotics is an increasingly common problem in both veterinary and human medicine, and its management is the subject of urgent debate^{1–4}. Efforts to reduce this resistance are based on the assumption that it is maintained in bacterial populations as a result of exposure to antibiotics, and that restricting the use of antibiotics should therefore restrain the spread of resistance. But we have found that antibiotic resistance is prevalent in populations of wild rodents that have not been exposed to antibiotics, indicating that approaches to control it based on this assumption may be overoptimistic.

The source of antibiotic-resistance genes is not always known. They can evolve within the treated host species⁵ or, frequently, like the genes encoding the antibiotics themselves, as a result of natural inter- and intraspecific bacterial competition^{3,6,7}. The

Table 1 Antibiotic resistance in enterobacteria isolated from faeces of wild rodents

Bacteria (no. of isolates)	Te	Tm	Na	Cm	Amc	Ap	Cxm
<i>Hafnia alvei</i> (41)	76 (4:4)	10 (0.5:0.5)	24 (1:64)	0 (0.5:2)	98 (> 64: > 64)	95 (> 64: > 64)	100 (64: > 64)
<i>Escherichia coli</i> (35)	14 (1:2)	0 (0.5:0.5)	9 (1:1)	0 (1:2)	97 (16:32)	89 (16:64)	100 (16: > 64)
<i>Serratia liquefaciens</i> (30)	63 (2: > 64)	30 (0.5:32)	30 (0.5:8)	0 (2:4)	100 (> 64: > 64)	97 (> 64: > 64)	90 (64: > 64)
<i>Alcaligenes spp.</i> (18)	44 (1:2)	67 (8: > 64)	56 (4:16)	0 (1:4)	67 (64: > 64)	67 (> 64: > 64)	78 (> 64: > 64)
<i>Serratia fonticola</i> (18)	50 (1:4)	0 (0.5:0.5)	22 (0.5: > 64)	0 (0.5:1)	72 (32: > 64)	94 (> 64: > 64)	67 (> 64: > 64)
<i>Enterobacter intermedius</i> (13)	39 (1:2)	23 (0.5:1)	23 (1:8)	0 (0.5:1)	85 (32: > 64)	92 (64: > 64)	77 (8: > 64)
<i>Enterobacter amnigenus</i> (10)	50 (1:4)	0 (0.5:0.5)	40 (1: > 64)	0 (1:1)	90 (32: > 64)	100 (> 64: > 64)	90 (> 64: > 64)
<i>Cedacae davisiae</i> (9)	44 (0.5:4)	22 (0.5:1)	22 (0.5: > 64)	0 (0.5:1)	67 (> 64: > 64)	89 (64: > 64)	89 (32: > 64)
<i>Providencia rustigianii</i> (6)	17 (1:1)	17 (0.5:0.5)	17 (1:1)	0 (1:2)	100 (32: > 64)	83 (64: > 64)	67 (32: > 64)

The prevalence of resistance is expressed as the percentage of isolates that are resistant (based on breakpoints). The degree of resistance in the population overall is given by the minimum inhibitory concentration for 50% and 90% of isolates (MIC₅₀ and MIC₉₀). Antibiotics: Te, tetracycline; Tm, trimethoprim; Na, naladixic acid; Cm, chloramphenicol; Amc, amoxycillin/clavulanic acid; Ap, amoxycillin; Cxm, cefuroxime.

expression of antibiotic resistance in bacteria may also involve a fitness cost that is disadvantageous compared with susceptible bacteria unless antibiotics are present in the environment^{2,4}, and resistance would gradually be lost from untreated populations. This idea is supported by some laboratory experiments and by a low prevalence (but, importantly, not absence) of resistance in human and animal populations not exposed to antibiotics^{8,9}.

If this is the case then improving the management of antibiotics should reduce the prevalence of resistance. However, compensatory mechanisms can evolve to overcome any small disadvantage of resistance in the absence of antibiotic selection, and resistance can be maintained in populations in the apparent absence of specific antibiotic selection for several years^{4,10–13}.

To test whether the development of antibiotic resistance is less prevalent in the absence of exposure to antibiotics, we surveyed resistance in commensal, enteric Enterobacteriaceae isolated from two wild populations of small rodents. Faeces from 38 bank voles (*Clethrionomys glareolus*) and 70 wood mice (*Apodemus sylvaticus*) were collected from two woodland sites on the Wirral peninsula in northwest England. The first wood is surrounded by more woodland, gardens and an ornamental lake; the second wood is surrounded by woods and by meadows in which heifers (but not dairy cows) are occasionally kept. Pheasant feed containing the antibiotic tylosin was sometimes distributed in the second site, but otherwise the rodents had minimal contact with antibiotics or with domestic animals routinely treated with them.

The most abundant enterobacterial isolate from each sample was screened for resistance using minimum inhibitory concentrations (MICs) of seven representative

antibiotics¹⁴, and the prevalence of resistance was calculated for 'breakpoint' MICs to reflect its clinical significance (at least for humans)¹⁴ (Table 1); only those species isolated six or more times are included, although the patterns were the same for less frequently isolated bacteria.

Overall, 90% of coliforms were resistant to amoxycillin, amoxycillin/clavulanic acid and cefuroxime. Depending on the bacterial species, 14–76% of coliforms were tetracycline resistant and 0–67% were trimethoprim resistant, but all of them were sensitive to chloramphenicol. Controlled disc diffusion was also carried out on six antibiotics (ampicillin, gentamicin, apramycin, sulphonomides, cefotaxime and cephadrine). Overall, 90% were ampicillin resistant, as a result of β -lactamase expression in more than half the cases (as determined by hydrolysis of a chromogenic cephalosporin, nitrocefin). The level of resistance to apramycin was low, at 12–33%, whereas resistance to the other antibiotics was comparable, at 33–73%.

Our results show that resistance to antibiotics is widespread in at least some wild populations, even though these have never to our knowledge been exposed to antibiotics, and they undermine the presumption that resistance will decline in the absence of antibiotic treatment. The origin(s) of the resistance and the selection mechanisms (if indeed such mechanisms are necessary) responsible for maintaining a high prevalence of resistance are unknown. It is important to address these questions, not least because similar mechanisms may operate in farm animals and humans, in which case the management of antibiotic resistance may need to be reconsidered.

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Sexual selection

Condition-related mate choice in sticklebacks

Exactly how the high genetic variance of traits involved in sexual selection through female mate choice is maintained is a much debated issue^{1–3}. Theoretical models attempt to explain the high genetic variance of sexual traits, among others, by the evolution of condition dependence^{1,4}, in which expression of the trait depends on physical condition. If it is costly to be choosy, then these models should also apply to female mate preference and predict that female condition affects the choice of mate. Here we allow female laboratory-bred three-spined sticklebacks, *Gasterosteus aculeatus*, to choose between computer animations of courting males⁵, and find that the choice of mate correlates well with female physical condition.

Heritability estimates of mate preference have accumulated in recent years: the average heritability ($\pm$ s.d.) of mate preference in 15 species was 0.41 ($\pm$ 0.21) (ref. 3), which is high for a behavioural trait⁶. Condition dependence might explain the high heritability of costly mate preferences, as it does the high genetic variance in sexual traits^{1,4}. Costs may include physiological as well as choice costs. Condition dependence of sexual traits is well known⁷, and there is some evidence for condition-dependent mate choice^{3,8}. We have studied the covariance of condition and mate preference in a fish species that is well-known for intensive sexual selection².

Sticklebacks were bred from wild-caught parents, and were raised and maintained

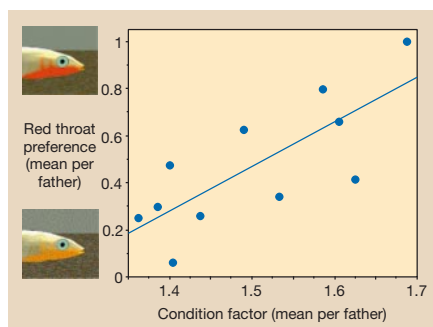


Figure 1 Relation between mean rearing condition (based on mean male winter condition per group) and mean red preference of ripe females from 11 different fathers. Females from one father were full siblings, except in three cases in which they had two different mothers, where the mean condition per father was weighted for the number of females tested per mother. Condition is calculated as $100 \times \text{body mass (g)} / \text{body length}^3$ (in cm) (ref. 9) and represents non-reproductive winter condition, estimated from data of 6 (range 2–9) randomly chosen males per full-sib group after one day of starvation. Mate preferences of 2 (range 1–5) ripe females per full-sib group were tested by using computer animations⁵. Females were given 2 min to choose between two 'virtual' males differing only in red throat coloration. We used standard colour chips to match the redness of the virtual males to those on standardized photographic slides of courting males. We selected bright red and bright orange males from the tails of the distribution of red intensity in the field based on densitometer-analysis¹⁰. Bright red and bright orange males differ mainly in hue (hue/saturation/brightness values of virtual males were 17°/100%/93% and 31°/97%/97%, respectively, as determined using Adobe Photoshop Version 4.0.1 software), probably because of differences in carotenoid composition¹¹. Male position was alternated between tests. Choice behaviour was video-recorded from above to avoid observer bias. Preferences were calculated as the time directed towards one male divided by the time directed to both males (0.5 indicates no preference; 1, absolute preference for the red male; 0, absolute preference for the orange male). Only females that spawned within 24h of the test with a live male were used.

under standardized laboratory conditions in full-sibling groups of similar size. Paternal effects on offspring traits were excluded by removing clutches from the fathers' nests one hour after fertilization. After reaching adult body size, fish were transferred from simulated summer to winter conditions, under which gonadal development and the development of male breeding coloration are suppressed.

Six months later, the condition factor⁹ of a random sample of males per full-sib group was assessed. Average male winter condition per group was taken as an index of group rearing condition because some females showed signs of egg production under the prolonged winter conditions. Male physical condition is not confounded by possible between-group variance in ovarian development. Females were randomly sampled from the full-sib groups about six months later and placed individually in 10-litre tanks under simulated summer conditions, where they became reproductive.

When females became ripe they were tested for their preference for red males by

placing them in a one-litre cuvette in front of a computer monitor on which two virtual males that differed only in red breeding coloration (bright red or bright orange) were courting simultaneously⁵. In this preference test, males differ in only one trait, and there are no costs of choice.

Despite standardized rearing conditions, progeny from different fathers differed significantly in condition during rearing (ANOVA, $F(10,67) = 7.65$, $P < 0.001$) and in preference for red coloration (ANOVA, $F(10,13) = 3.52$, $P < 0.02$). Overall, there was no significant preference for the bright red male over the bright orange one (t -test, $t = 0.37$, $df = 10$, $P > 0.71$), but average red preference and rearing condition per father correlated positively ($r = 0.77$, $N = 11$, $P < 0.01$; Fig. 1). Females from groups with a high average condition factor before reproduction therefore preferred the red male, whereas those of lower rearing condition showed a preference for the orange male.

Combined with condition dependence of sexual traits, the covariance of mate preferences with condition may have consequences for the evolution of both preferences and preferred sexual traits. Experiments must show whether mate preferences have condition-dependent expression. When mate preferences appear to be condition dependent, and assuming there is high genetic variance in condition⁴, pleiotropic effects of genes that influence condition would be expected to lead to high genetic correlations between sexual trait and mate preference. Alternatively, the covariance of mate preference with condition may be brought about by genetic covariance of sexual male traits with both female preference¹⁰ and male condition. The consequences of these alternatives should be explored using theoretical models of sexual selection.

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Structure of the *trp* RNA-binding attenuation protein, TRAP, bound to RNA

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The *trp* RNA-binding attenuation protein (TRAP) regulates expression of the tryptophan biosynthetic genes of several bacilli by binding single-stranded RNA. The binding sequence is composed of eleven triplet repeats, predominantly GAG, separated by two or three non-conserved nucleotides. Here we present the crystal structure of a complex of TRAP and a 53-base single-stranded RNA containing eleven GAG triplets, revealing that each triplet is accommodated in a binding pocket formed by β -strands. In the complex, the RNA has an extended structure without any base-pairing and binds to the protein mostly by specific protein–base interactions. Eleven binding pockets on the circular TRAP 11-mer form a belt with a diameter of about 80 Å. This simple but elegant mechanism of arresting the RNA segment by encircling it around a protein disk is applicable to both transcription, when TRAP binds the nascent RNA, and to translation, when TRAP binds the same sequence within a non-coding leader region of the messenger RNA.

Attenuation¹ is one of several mechanisms regulating the transcription of bacterial operons. This mechanism acts during transcription of the 5' leader regions preceding the structural genes, by folding the nascent RNA into alternative base-paired hairpin structures, one of which signals RNA polymerase to stop transcription. In *Bacillus subtilis* and several other bacilli, the attenuation of the tryptophan biosynthetic genes is controlled by TRAP, which senses the intracellular levels of the amino acid^{2,3}. TRAP binds a specific RNA sequence in the leader segment of the nascent RNA transcript, but only when activated by bound L-tryptophan^{4,5}. The RNA target for TRAP contains eleven trinucleotide repeats, almost exclusively made up of GAG or UAG triplets, separated by two or three variable 'spacer' nucleotides^{6,7} (Fig. 1). By binding to this RNA, TRAP disrupts or prevents the formation of an 'antiterminator' stem-loop structure, allowing formation of an alternative 'terminator' hairpin that leads to termination of transcription⁸.

B. subtilis has no *trp*-repressor like that controlling transcription initiation in *E. coli*, and transcription is solely regulated by attenuation. The basic principle of attenuation, the folding of RNA into two alternative hairpin structures, one of which terminates transcrip-

tion, is the same in *B. subtilis* and *Escherichia coli*. However, the strategies for achieving this are different. Unlike *B. subtilis*, *E. coli* and other enterobacteria have no TRAP protein: transcription attenuation is controlled by the movement of the ribosome along the messenger RNA region encoding a tryptophan-rich leader peptide, and the position of the ribosome (governed by L-tryptophan levels) dictates RNA secondary structure¹.

Even when the *B. subtilis trpEDCFBA* mRNA is fully transcribed, TRAP binding to the same site in the non-coding leader region of mRNA can regulate translation of *trpE*^{9,10}. Here, TRAP binding causes the downstream RNA to refold, sequestering the *trpE* ribosome-binding site in an RNA hairpin and thus inhibiting ribosome binding^{9–11}. TRAP also regulates translation of *trpG*, which is located in a folic acid biosynthetic operon¹². In this case the Shine–Dalgarno sequence is within the TRAP binding site^{6,13}, hence TRAP directly competes with the ribosome to bind RNA.

The TRAP molecule in complex with L-tryptophan has been crystallized and its three-dimensional structure determined^{14,15}. It is an eleven-subunit symmetrical protein of relative molecular mass ~91,000 ($M_r \approx 91K$) whose subunits are arranged in a ring. The

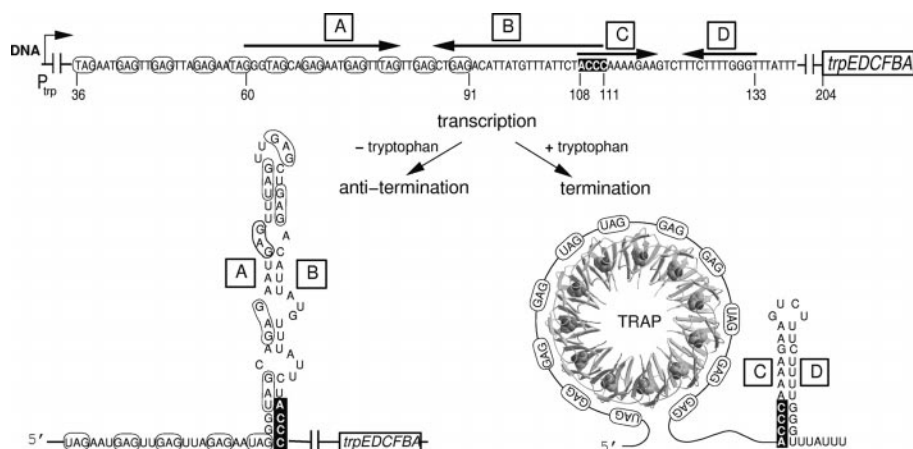


Figure 1 Transcription attenuation in bacilli². The 203-nucleotide leader region that precedes the structural genes of the *trpEDCFBA* operon of *B. subtilis* is shown with numbering corresponding to the start of transcription. The inverted repeats predicted to form the terminator (nucleotides 108–133) and the antiterminator (nucleotides 60–111)

stem-loops in the RNA transcript are designated by large boxed letters with complementary strands shown by arrows above the DNA. GAG and TAG triplets (or UAG within RNA transcripts) are circled. TRAP is shown as a ribbon model bound to the 36–91 region of the RNA transcript containing the eleven GAG and UAG triplets.

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Table 1 X-ray structure statistics

Data collection		
Resolution range (outer shell)	30.0–1.9 Å (1.93–1.90 Å)	
No. of independent reflections	154,404	
Redundancy*	3.3 (2.1)	
Completeness (%)	96.4 (71.7)	
$\langle I/\sigma(I) \rangle$	11.8 (2.4)	
$R_{\text{merge}}^\dagger$	0.088 (0.396)	
.....		
Refinement and model correlation		
No. of reflections used in refinement	152,857	
R -factor‡	0.188	
No. of reflections used for R_{free}	1,545	
$R_{\text{free}}^\ddagger$	0.235	
No. of protein atoms	12,176	
No. of RNA atoms	1,269	
No. of water molecules	1,210	
Average B -factor (Å ²)	29.5	
R.m.s. deviations from ideal geometry (target values are given in parentheses)		
Distances, Å	Bond lengths (Å)	0.010 (0.020)
	Bond angles (Å)	0.026 (0.040)
Non-crystallographic symmetry (Å)§		
	Protein main chain	0.059 (0.1)
	RNA atoms	0.168 (0.3)

* The average number of observations of the same reflection.

† The value of the merging R -factor between equivalent measurements of the same reflection, $R_1 = \sum |I - \langle I \rangle| / \sum I$.

‡ Crystallographic R -factor, $R_{\text{free}} = \sum ||F_o| - |F_c|| / \sum |F_o|$.

§ R.m.s. deviation of atoms of the 11 subunits from the average structure.

structure shows that each of the 11 L-tryptophans is bound between adjacent TRAP subunits with every possible hydrogen bond made and with the indole moiety buried inside an intersubunit hydrophobic pocket. The circular assembly of TRAP subunits indicated that, upon binding, the 11 G/UAG trinucleotides of the RNA might encircle the protein. This prediction was supported by site-directed mutagenesis¹⁶, which mapped RNA-binding residues onto the outer perimeter of the protein ring.

Here we report the crystal structure of TRAP from the thermophilic bacterium *Bacillus stearothermophilus* in complex with a 53-base single-stranded RNA molecule, determined with data extending to 1.9 Å spacing. There are several distinct features of this complex. Most of the protein interactions with RNA are to the bases and there are no direct interactions with phosphates. Also, the RNA itself does not fold into an organized secondary structure but instead has an extended conformation wrapping itself around the protein molecule. Inevitably, the structure has a repetitive character, the eleven GAG triplets interacting and mapping onto the 11-fold related binding sites on the protein.

Overall structure in the crystal

Crystals of TRAP in complex with a 53-base RNA, containing eleven GAG triplets separated by AU dinucleotides, were grown from a 2:1 molar ratio of TRAP 11-mers to RNA. The crystals belong to the space group $C2$ with $a = 151.0$ Å, $b = 111.7$ Å, $c = 138.7$ Å and $\beta = 117.8^\circ$. The structure was determined by molecular replacement using the structure of native TRAP¹⁷ and refined with data extending to 1.9 Å spacing (Table 1). In the structure, the eleven triplets and ten separating AU dinucleotides that constitute the 53-base RNA molecule are arranged in a circle around the perimeter of

the TRAP 11-mer (Fig. 2a). The eleven GAG triplets interact with eleven binding sites on the surface of TRAP; each binding site is built up by residues from two adjacent subunits (Fig. 2b).

In the crystals, the TRAP 11-mer assembles into discrete columns of four molecules in which only the central two bind RNA (Fig. 2c). The two end molecules make strong crystal contacts through their potential RNA-binding surfaces. In contrast, the barrel surfaces of the two central molecules are completely exposed to the solvent and each binds a 53-base RNA segment which wraps around the protein molecule like a belt (Fig. 2a). If we designate the narrower end of the TRAP molecule as the 'head' and the wider end as the 'tail' then we can refer to the packing of the two top (or the two bottom) molecules of the column (the two molecules constitute the asymmetric part of the crystal) as head-to-tail. The crystallographic two-fold symmetry brings two pairs of these molecules together to make a cylinder of four molecules, with the central pair oriented tail-to-tail. The packing of the TRAP–RNA complex is distinctly different from that observed in crystals of RNA-free TRAP^{15,17}. RNA binding is probably not the cause of this four-molecule assembly, as each RNA binds only one TRAP 11-mer and makes no other contacts. It seems probable that the tetrameric assembly in which only two 11-mers bind the RNA is a consequence of the crystal lattice and does not reflect functional importance. In agreement with this, dynamic light scattering with *B. stearothermophilus* TRAP indicates a 1:1 molar ratio for the TRAP–RNA complex in solution (A.A.A. & P.G., unpublished results).

RNA conformation

In the crystal structure of the TRAP–RNA complex, the repetitive GAGAU segments have essentially the same three-dimensional structure, although there are small differences between the conformational angles of individual GAGAU segments (Table 2). These differences reflect the flexibility of the protein–RNA interface (see below). The gap between the first and last GAG triplet is not detected in the electron density of the crystal; the missing spacer between the first and the eleventh triplet is randomized over the eleven sites, implying that the electron density at each corresponds to the spacer dinucleotides occurring only 10 out of 11 times.

RNA frequently forms double-stranded or hairpin helical structures with phosphate-sugar torsion angles and sugar pucker very similar to those of A-DNA¹⁸. This conformation also appears to be favoured in the TRAP–RNA structure, even though the RNA is in a single-stranded extended state. In the complex, the central three nucleotides of the GAGAU repeats (A2, G3, A4) have main-chain torsion angles and furanose ring conformations similar to those of A-DNA (Table 2, Fig. 3).

In contrast, the two outer nucleotides of the GAGAU repeating sequence, G1 and U5, have different conformational parameters to those of A-DNA (Table 2). Similar conformational angles to these have been observed for single nucleotides in loop regions of RNA hairpins^{19,20} and turn regions of transfer RNA²¹. In TRAP RNA, such angles are observed in two consecutive nucleotides, the U5 and G1 of the next five-nucleotide repeating unit. These two nucleotides form a turn that could be envisaged as a short piece of a left-handed helix, compensating for the right-handed twist in the AGA repeats. Thus

Table 2 RNA geometry

Nucleotide	Sugar pucker	α	β	γ	δ	ϵ	ζ	χ	Weighted average difference
G1	C2'-endo	341 (16)	163 (8)	20 (10)	145 (5)	244 (3)	88 (4)	247 (4)	5.34
A2	C3'-endo	278 (6)	157 (3)	24 (3)	95 (3)	213 (3)	273 (2)	265 (2)	1.43
G3	C3'-endo	293 (4)	159 (2)	55 (3)	91 (3)	214 (3)	276 (3)	256 (3)	0.82
A4	C3'-endo	302 (10)	189 (4)	37 (7)	92 (4)	213 (9)	303 (10)	210 (10)	1.10
U5	C2'-endo	311 (18)	179 (5)	48 (17)	137 (10)	204 (6)	134 (13)	257 (7)	3.90
A-DNA	C3'-endo	293 (17)	174 (14)	56 (14)	81 (7)	203 (12)	289 (12)	199 (8)	–

For the torsion angles, mean values over eleven observations are shown with r.m.s. deviations from the mean value given in parentheses. For comparison, torsion angles for A-DNA are also given¹⁷. The weighted average difference was calculated between sugar-phosphate conformational angles of TRAP–RNA and those of A-DNA.

the 55-mer is stitched around the TRAP surface with alternating A-form helical triplets separated by left-handed dinucleotide turns.

If an RNA helix were built up with the U5:G1 dinucleotide angles it would, like Pauling's early predicted DNA structure²² and like the stretched and overwound P-DNA²³, have bases situated outside the internal sugar-phosphate backbone. In contrast to these two structures, however, the U5:G1 helix is left-handed and single-stranded with about 6.5 bases per turn, and an internucleotide distance along the helix of about 3.0 Å. The U5:G1 sugar-phosphate backbone conformation is stabilized by a number of RNA-protein interactions (see below), perhaps indicating that it is not energetically

favourable on its own.

The base conformations are generally quite different to those of an A-DNA helix and are largely dictated by interactions with the protein. Only A4 is close to the classical *anti* conformation (Table 2). The two other nucleotides, A2 and G3, have their bases stacked together (Fig. 3) but with χ -torsion angles differing from the classical values by about 60° (Table 2). The bases of A4 and U5 are also stacked, in spite of their non-helical backbone conformation; this is achieved by rotation of the U5 base by about 50° relative to A4. G1 is not stacked on any other bases but instead with the aliphatic atoms of the side chain of Lys 37.

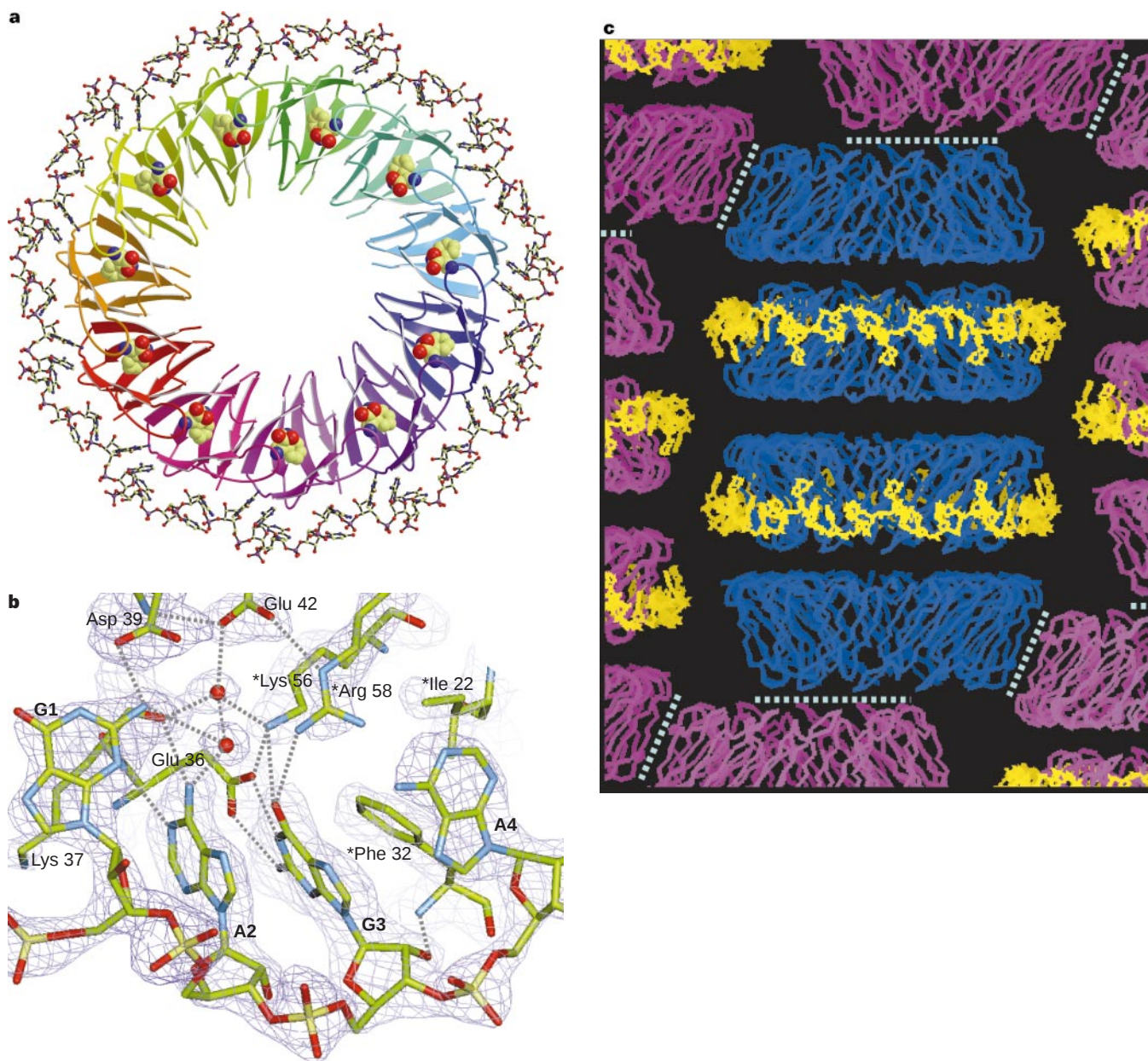


Figure 2 Overall structure of the TRAP-RNA complex. **a**, The ribbon diagram of one TRAP 11-mer complexed with RNA, viewed along the 11-fold symmetry axis. Each subunit is shown in a different colour. RNA is shown in ball-and-stick models and L-tryptophan molecules are shown as van der Waals spheres. **b**, Electron-density maps at the RNA-binding site calculated with likelihood-weighted $2|F_o| - |F_c|$ coefficients. The refined model is shown with hydrogen bonds as dashed lines and water molecules as red spheres; asterisk designates residues from one of the two subunits forming the RNA-binding cleft. Oxygen, nitrogen, carbon and phosphorus are shown in red, blue, lime

green and yellow, respectively. The view is from outside the molecule towards the RNA-binding surface. **c**, Packing of molecules in the crystal. RNA is in yellow, the four protein molecules that constitute one cylinder are represented as blue ribbons and surrounding protein molecules as pink ribbons. The four blue molecules are oriented head:head:-tail:tail (from top to bottom) around the vertical 11-fold symmetry axis. Areas of crystal contacts are shown by broken lines. **a** was generated with BOBSCRIPT^{48,49} and Raster3D⁵⁰; **b** and **c** were generated by QUANTA (Molecular Simulations).

Protein–RNA interactions

The presence of eleven chemically identical copies of protein–GAGAU units in the asymmetric unit of the crystals provides eleven independent observations of protein interactions with the GAGAU repeating unit. Differences in these interactions at the protein subunits reflect the dynamics of the protein–RNA interface, the eleven observations providing snapshots of possible conformations (Fig. 4a). The largest differences are observed for the basic side chains of Lys 37 and Arg 58, with r.m.s. deviations of 0.43 and 0.45 Å, respectively, from the average structure. Both residues make direct interactions with the RNA, and changes in their side-chain conformations induce small changes in the position of the RNA itself. The description of protein–RNA interactions given below is for the most populated conformations, with some details of observed conformational differences.

The G1 base packs against the TRAP surface making non-polar contacts with the extended aliphatic chain of Lys 37, whose ε-amino group makes a stabilizing hydrogen bond with Asn 20 (Fig. 4b). The NH₂ of guanine makes a distorted hydrogen bond with the carboxyl oxygen of Asp 39. The sugar-phosphate moiety makes no contacts with the protein.

A2 makes three hydrogen bonds with the protein. One of these is from the sugar hydroxyl to a bridging water molecule, which is hydrogen bonded to the main-chain carbonyl oxygen of Thr 30 (Fig. 4b). The other two hydrogen bonds are made by the base. The exocyclic amino group of adenine is hydrogen bonded to the main-chain carbonyl oxygen of Lys 37 and N1 accepts a hydrogen bond from the Lys 37 main-chain NH. The adenine base is stacked nearly parallel against the following guanine, which is in turn stacked against the side chain of Phe 32.

G3 is bound to TRAP through six hydrogen bonds, five of which are made by the base (Fig. 4b). The carbonyl oxygen of the G3 base hydrogen bonds to the Nε of Lys 56 and, in eight of the eleven subunits, to the side-chain NH₂ of Arg 58. The side-chain carboxyl

of Glu 36 accepts a pair of hydrogen bonds from two nitrogen functions, N1 (NH) and N2 (NH₂) of the base. The NH₂ of guanine is hydrogen bonded to the main-chain carbonyl oxygen of Thr 30. In addition, there are several water-mediated hydrogen bonds. The guanine base is buried in a pocket formed by the adjacent adenosine and Phe 32. The ribose 2' hydroxyl makes a hydrogen bond to the main-chain NH of Phe 32.

A4 and U5 have their bases stacked in van der Waals contact with each other (Fig. 4c) and in general make little contact with the protein, consistent with observations that the spacer nucleotides are not conserved in nature¹⁷ and their composition is not crucial for binding^{7,24}. The only hydrogen bond is from the A4 sugar hydroxyl to a water molecule that is hydrogen bonded to the main-chain nitrogen and carbonyl oxygen of Ser 35. Additionally, in several subunits there are hydrogen bonds from bases to water molecules, but these vary in detail.

Specificity of RNA–TRAP interactions

When activated by binding L-tryptophan, TRAP binds its cognate RNA with high affinity ($K_d \approx 10^{-10}$ M; ref. 25). Optimal binding requires that the repeated triplets are separated by two spacer nucleotides²⁶. Sequence analysis of leader regions from five different bacilli shows 70% GAG, 26% UAG and only one each of AAG and CAG triplets¹⁷. The second base is always adenine and the third is always guanine. These two bases are bound to TRAP through a set of intensive interactions (Fig. 4b) which determine the specificity. In contrast, the base of the first nucleotide is variable. In the structure of the TRAP–RNA complex, the G1 base is stacked to the Lys 37 side chain and makes a single hydrogen bond to Asp 39. Conformational searches show that the unusual torsion angles of the sugar-phosphate backbone at G1 (Table 2) generate a penalty of the order of 10 kcal mol⁻¹, equivalent to 2–3 hydrogen bonds.

Molecular modelling with RNA segments in which the first nucleotide is G, U, A or C indicates that all bases would make

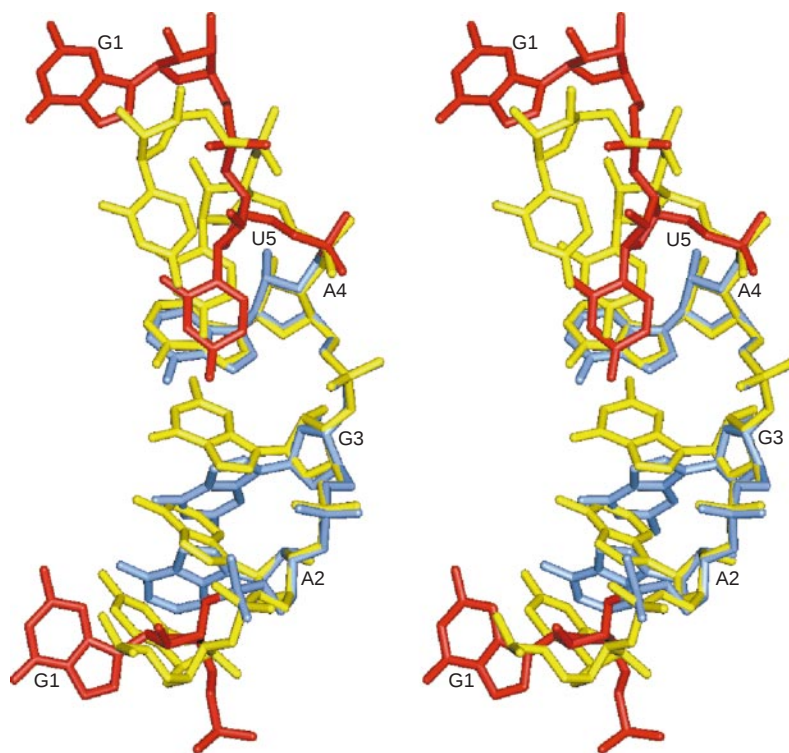


Figure 3 RNA conformation. Stereo diagram of one GAGAU unit and G of the next five-nucleotide repeating unit of RNA in the TRAP–RNA complex with the A-form AGA helical

triplet in blue and U5:G1 nucleotides, forming the left-handed turn, in red. The structure is overlapped with a 6-nucleotide segment of A-form helical RNA (shown in yellow/green).

similar stacking interactions with the side chain of Lys 37, possibly compensating for the penalty. A hydrogen bond to Asp 39 would be preserved only for guanine. This hydrogen bond, however, does not appear to be crucial for the stability of the entire TRAP–RNA complex as, in the *B. subtilis* system, Asp 39 can be replaced by Ala without a significant loss of binding¹⁶. In contrast, the stacking interaction appears to be important, as replacement of Lys 37 by Ala

significantly reduces RNA binding¹⁶. Molecular modelling detected a preference for guanine, but did not distinguish between uracil, adenine or cytosine at the first position. Filter-binding assays do not discriminate between G, A, U and C at the first position of the five-nucleotide repeat in RNAs with eleven identical repeats²⁷.

Therefore, the appearance of guanine and uracil in position 1 of the triplet repeats in natural RNA sequences does not appear to be

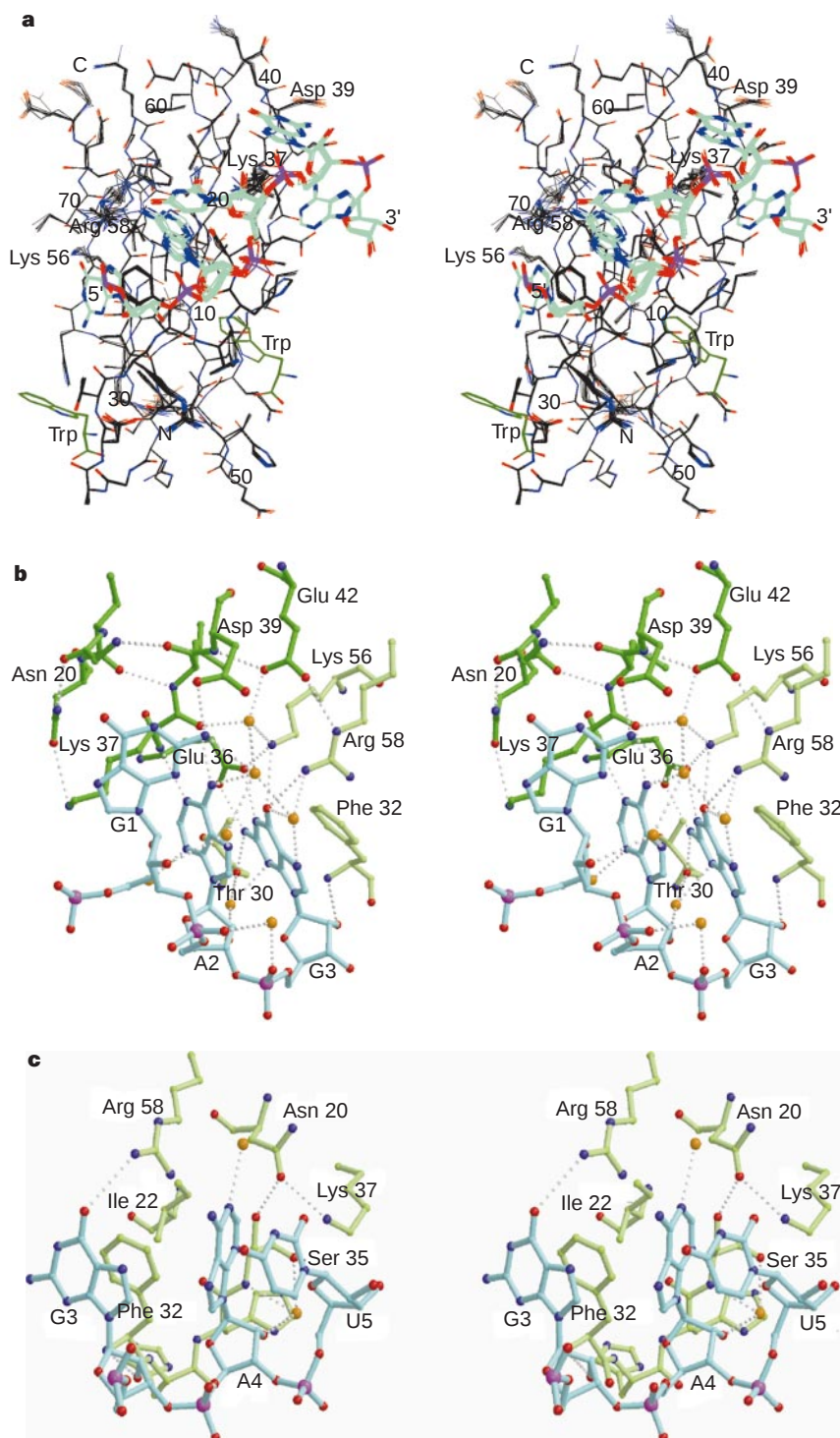


Figure 4 Protein–RNA interactions. **a**, MOLSCRIPT⁴⁸ stereo plot of 11 crystallographically independent subunits of TRAP with corresponding RNA segments overlapped by least-squares fit of protein main-chain atoms. Every tenth residue is numbered; carbon atoms of protein, RNA and activating L-tryptophan are black, aquamarine and green, respectively. **b**, Interactions with G1, A2 and G3. Bonds in ball-and-stick models are aquamarine

for RNA atoms and dark green or light green for atoms from the two adjacent protein subunits. Hydrogen bonds are shown by dashed lines, water molecules as orange spheres, and atoms of oxygen, nitrogen and phosphorus are shown in red, blue and purple, respectively. **c**, TRAP interactions with A4 and U5.

correlated with the affinity of TRAP–RNA interactions. The basis for the preference of guanine and uracil may arise from events involving the triggering of undesirable intramolecular base-pairing in the full-length RNA. Alternatively, the presence of G and U in the first position may reduce the degree of conformational freedom during binding of RNA to TRAP. U, like G, and unlike A or C, has an imino proton on the ring nitrogen, which may fulfil the hydrogen-bonding potential of Asp 39 and could therefore accelerate RNA binding.

The repetitive character of the GAG–protein interactions in the TRAP–RNA complex will facilitate the binding of adjacent repeats by effectively increasing the local concentration of the RNA target. This indicates that the binding may be cooperative, as confirmed by preliminary assays with RNA targets containing different numbers of trinucleotide repeats (M. Elliott and P.G., unpublished results).

Structure–function relationships

Each polypeptide chain of TRAP has to perform at least three different functions. First, it has to fold and form a circular assembly with the ten other subunits; second, it has to bind L-tryptophan, which is accommodated in deep binding clefts¹⁵; and third, it has to bind RNA. Large surface areas (2,489 Å² and 414 Å²) of each subunit become buried in intersubunit contacts and upon RNA binding, respectively. Given the small size of each polypeptide (75 amino acids), it is not surprising that some amino acids are simultaneously involved in several functions. One example of such a multifunctional residue is Glu 36. Replacing this residue with Ala disrupted tryptophan binding¹⁶, indicating its importance for proper protein folding. As seen from the structure, Glu 36, together with Phe 32 and Lys 37, presents crucial interactions to the bound RNA triplet and is a key determinant of specificity and high affinity. Substituting Lys 56 and Arg 58 with alanine did not affect L-tryptophan binding, but reduced RNA binding¹⁶. From the structure we see that the charged functions of these two residues are in hydrogen-bonding interactions with the G3 base (Fig. 4b); but, perhaps more importantly, the aliphatic parts of these two residues determine the shape of the RNA-binding pockets.

Replacing Phe 32 with Ala did not affect the affinity of *B. subtilis* TRAP for RNA¹⁶, indicating that the stacking of the A2 and G3 bases with the Phe 32 ring may not be necessary for binding. However, the conservation of Phe 32 in known TRAP sequences¹⁷ indicates that it could be involved in determining the specificity of TRAP interactions by excluding other bases. The hydrogen bond for the main-chain NH of Phe 32 to the G3 ribose 2' hydroxyl appears to be critical, as RNA analogues with deoxyribose at the G3 position of all eleven repeats exhibit at least 8×10^3 -fold weaker binding to TRAP²⁷. For Lys 37 there are well defined van der Waals contacts between the aliphatic atoms and the base (Fig. 4b), providing a nearly ideal surface for the packing and conformation of the aromatic ring. This role of the Lys 37 side chain has been confirmed by replacement with Met or Phe, both of which maintain satisfactory binding (A. Wendt and P.G., unpublished results).

Regulation of attenuation by TRAP

It is now possible to follow the structural events leading to attenuation of transcription by TRAP. The first event is the binding of L-tryptophan to the TRAP 11-mer ($S_{0.5} = 10^{-5}$ M; ref. 16). Following the arrival of the L-tryptophan, loop residues 25–33 bury the amino acid¹⁵ making a series of main-chain and side-chain interactions that stabilize the local conformation. Once the loop is fixed, Phe 32 becomes able to form critical contacts with RNA through its main-chain NH. In addition, other residues that are critical for binding RNA, such as Glu 36 and Lys 37, are adjacent to this loop and might also be affected by L-tryptophan binding. During transcription, when the leader region of the *trpEDCFBA* mRNA is being synthesised by RNA polymerase, the TRAP–L-tryptophan complex binds nucleotides +36 to +91 of the nascent

RNA⁹. The circular arrangement of the TRAP subunits generates a perfect matrix for the binding of the polymeric RNA molecule, which forms a belt around TRAP (Fig. 2a) with the GAG and UAG trinucleotide repeats accommodated in binding pockets. In the complex, all bases of RNA are stacked, either to an adjacent base or to a protein side chain; stacking with the protein side chain compensates for the loss of the helical base–base interactions. RNA binding is accompanied by conformational changes in the side chains of Phe 32, Lys 37, Asp 39, Lys 56 and Arg 58. The side chains of these residues are largely disordered in the absence of RNA, but all adopt well-defined conformations in the complex, interacting directly with the RNA. The RNA region becomes arrested by TRAP and unable to participate in folding of the antiterminator hairpin, therefore allowing the region +108 to +133 to fold into the terminator hairpin, which signals RNA polymerase to stop transcription. Similar TRAP–RNA interactions occur during regulation of the translation of *trpE* and *trpG*. In both cases, TRAP encircles the particular RNA region around the protein disk, sequestering the ribosome binding site either directly, as in the case of *trpG*⁹, or indirectly (*trpE*), by causing the downstream RNA to refold^{6,13}.

Protein interactions with RNA

Proteins often interact with single-stranded nucleic acids through β -sheet surfaces. Ribonucleoprotein domains bind single-stranded RNA segments at the surface of the four-stranded antiparallel β -sheet^{19,28,29}. Replication protein-A binds single-stranded DNA at the surface of a channel formed by a β -sheet³⁰. TRAP also forms its RNA-binding surface using β -strands. However, there is a major difference: RNA binds not along the β -sheet surface, as in the other structures, but across the edges of the eleven β -sheets.

RNA binding often induces conformational changes in proteins. In some systems, such as MS2 bacteriophage coat protein, these changes occur in only a few side chains²⁰. In the case of RNA binding to U1A protein, there are significant conformational changes in the carboxy-terminal helix, which rotates away, opening the RNA-binding surface³¹. The 14-amino-acid RNA-binding segment of Tat from HIV is unstructured, but on binding to RNA adopts a β -turn-like conformation³². TRAP has essentially the same backbone structure with and without RNA: the r.m.s. difference between the main-chain atom positions is only 0.39 Å. However, RNA binding is associated with substantial conformational changes in the side chains of Phe 32, Lys 37, Asp 39, Lys 56 and Arg 58 (data not shown).

A striking feature of protein interactions with nucleic acids is the presence of salt bridges and hydrogen bonds with the sugar-phosphate backbone. TRAP presents a number of positively charged residues on its surface, in particular Lys 37, Lys 56 and Arg 58, which interact directly with RNA. However, there are no direct interactions with phosphates in the TRAP–RNA complex and few interactions with RNA sugars; the only direct hydrogen bond is formed between G3 sugar 2' hydroxyl and the Phe 32 main-chain nitrogen. Thus binding is heavily dependent on the base sequence of RNA.

At present there is relatively little structural information describing protein–RNA interactions. Moreover, the data are mostly from protein complexes with RNA molecules whose conformations are restricted by secondary structures such as stem-loop^{19,20,28} or the more complex cruciform of tRNA^{21,33,34}, or the structure seen in the 58-nucleotide fragment of the 23S rRNA^{35,36}. Except for the loop regions of several stem-loop RNAs, and for the uridine-rich segment of a transformer pre-mRNA intron of *Drosophila*²⁹, no structural information is currently available for protein interactions with extended single-stranded RNA molecules, and in particular with mRNA, interactions that are central to the processes of transcription and translation. Our analysis provides new insight into these interactions. One conclusion is that the recognition of

single-stranded RNA does not necessarily involve protein interactions with the phosphates of RNA. In addition, the structure shows that some RNA segments are bound to protein with the same conformational angles that occur in the A-form double helix; this indicates that TRAP may select from the nascent RNA transcript a favoured conformation that is likely to be predominant before binding. The regular alternation of helical and non-helical conformational angles of RNA on the TRAP surface is a consequence of its circular and repeating structure. Other protein complexes with single-stranded RNA may exhibit this alternation of conformations, but obviously not with the regular pattern seen in TRAP.

The remarkable symmetry in the TRAP system has not yet been seen in the attenuation mechanisms of other organisms^{37,38}. Despite possible advantages of its striking periodic character, other structural mechanisms have also been selected by evolution to control biosynthesis. Comparison of the structures involved in other attenuation systems may reveal the advantages and disadvantages of circular symmetry and establish general principles that govern protein–RNA interactions in biological processes. □

Methods

RNA synthesis

The 53-base RNA (GAGAU)₁₀GAG was synthesized by *in vitro* transcription with T7 RNA polymerase using BstNI linearized plasmid pSP64GAGAU as a template. This plasmid contains 10 repeats of GAGAT followed by GAG downstream of the T7 promoter as an EcoRI–Bsp120I fragment in pSP64/p36T (ref. 39). To create the insert, two oligonucleotides (5′-AGGAATTCATTATAATACGACTCACTATA-3′ and 5′-GAGGGCCC (ATCTC)₁₁TATAGTGAGTCG-3′) were annealed and the 3′ ends were extended using the Klenow fragment of DNA polymerase I. Transcription produced a (GAGAU)₁₀GAG–tRNA fusion RNA which was cleaved 5′ to the tRNA with the M1 RNA of RNase P (ref. 39). The RNA was gel-purified as described²⁵.

Crystallization and data collection

TRAP from *B. stearothermophilus* was overexpressed in *E. coli* and purified as described¹⁷. The protein complex with (GAGAU)₁₀GAG RNA was monitored by protein mobility shift in non-denaturing 7.5% polyacrylamide gels. Crystals grew from 2:1 and higher TRAP:RNA molar ratios, but not from a 1:1 molar ratio. The protein–RNA complex (~15 mg ml⁻¹), crystallized by the hanging-drop method, was in a solution containing 50 mM K-phosphate, pH 7.8, and 7.5 mM L-tryptophan. The reservoir contained 0.2 M K-glutamate, 50 mM Triethanolamine, pH 8.0, 10 mM MgCl₂ and 9–12% monomethyl ether poly(ethylene) glycol (PEG) 2000 (ref. 40). Crystals grew in two different forms. One crystal form belongs to the space group P21212 with very similar *a* and *b* cell dimensions (*a* = 146.5 Å, *b* = 146.5 Å, *c* = 158.5 Å). This form has three TRAP molecules in the asymmetric unit packed in a column, of which only the central molecule contains bound RNA. These crystals were not used for structure determination as the central molecule exhibits a mixture of two opposite orientations. The structure was determined using the second crystal form, which belongs to the space group C2 with *a* = 151.0 Å, *b* = 111.7 Å, *c* = 138.7 Å, *β* = 117.8 Å. These crystals grew within 2 days and retained useful diffraction quality for a further 1–2 days only. This necessitated that crystals be rapidly frozen in cryoprotectant buffer (containing 20% MPD). We collected X-ray data (Table 1) at 120 K using synchrotron radiation on the EMBL/Hamburg BW7B beamline (DESY, DORIS storage ring) at a wavelength of 0.84 Å with a 345-mm MarResearch image-plate scanner as detector. Data were processed using the programs DENZO and SCALEPACK⁴¹.

Structure determination and refinement

All crystallographic calculations were performed using the CCP4 program package⁴². For molecular replacement calculations, we used AMoRe using the 11-subunit molecule of *B. stearothermophilus* TRAP (Protein Data Bank⁴³ accession code 1QAW). Two solutions were found that, after rigid body refinement in the resolution range 10–3.0 Å, gave a correlation of 75.9% and R-factor of 36.1%. Refinement was performed using REFMAC⁴² with the positional and B-factor shifts damped by 50%. For model building we used the program XFIT⁴⁴ implemented in QUANTA (Molecular Simulations). The validity of the refinement and the model rebuilding process were monitored using *R*_{free} (ref. 45). During the refinement, non-crystallographic symmetry restraints were imposed on the protein main-chain atoms of the two 11-subunit molecules and on the RNA atoms of the 11 repeating units. The final electron-density maps allowed the positioning of all residues except the three amino-terminal residues of all 11 subunits in RNA-binding TRAP, five N-terminal residues of all 11 subunits in non-complexed TRAP and one or two C-terminal residues, depending on the particular subunit. Apart from L-tryptophan molecules bound to TRAP, there is one additional L-tryptophan that mediates crystal contacts between the columns of four 11-subunit TRAP molecules.

Molecular modelling

All calculations were performed using the CHARMM version 22 polar hydrogen force field⁴⁶ using a distance-dependent dielectric with a switching function operating between

10.5 and 11.0 Å to truncate the non-bonded interactions. Conformational searches for the free RNA backbone at the first G of the second GAGAU unit were performed in the (GAGAU)₂ decamer. We constructed four different RNA (XAGAU)₂, containing G, A, C and U at the first position of each of the two RNA repeats, using Quanta (Molecular Simulations). The terminal residues of the RNA model were held fixed to simulate the fact that the RNA maintains the looped structure while bound to TRAP. Four different models, including two adjacent subunits of TRAP, the associated RNA and bound L-tryptophan molecules, but not explicit solvation, were subjected to several minimization regimes which included 500 cycles of steepest descent followed by 2000 cycles of ABNR minimization. All regimes produced essentially similar results.

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Correspondence and requests for materials should be addressed to A.A.A. (e-mail: fred@york.ac.uk). The structure factors and the refined coordinates of the TRAP–RNA complex have been deposited with the Protein Data Bank⁴³; the accession codes are R1C9SSF and 1C9S, respectively.

The novel Cer-like protein Caronte mediates the establishment of embryonic left–right asymmetry

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In the chick embryo, left–right asymmetric patterns of gene expression in the lateral plate mesoderm are initiated by signals located in and around Hensen's node. Here we show that Caronte (Car), a secreted protein encoded by a member of the *Cerberus/Dan* gene family, mediates the *Sonic hedgehog* (*Shh*)-dependent induction of left-specific genes in the lateral plate mesoderm. Car is induced by Shh and repressed by fibroblast growth factor-8 (FGF-8). Car activates the expression of *Nodal* by antagonizing a repressive activity of bone morphogenic proteins (BMPs). Our results define a complex network of antagonistic molecular interactions between Activin, FGF-8, Lefty-1, Nodal, BMPs and Car that cooperate to control left–right asymmetry in the chick embryo.

Many of the cellular and molecular events involved in the establishment of left–right asymmetry in vertebrates are now understood. Following the discovery of the first genes asymmetrically expressed in the chick embryo¹, a model of left–right determination involving a complex cascade of genetic interactions has emerged^{2–4}. The model explains how inductive signals initiated in or near the organizer region of the gastrulating embryo control the establishment of asymmetric patterns of gene expression throughout the lateral plate mesoderm (LPM). These asymmetric patterns are interpreted during development to give rise to left–right asymmetries of internal organs and other embryonic structures.

Nodal is crucial in establishing initial left–right cues in the vertebrate embryo. The expression of *Nodal* in the left LPM is strictly correlated with the development of normal organ *situs* in all vertebrates examined so far^{5–7}. Furthermore, misexpression of *Nodal* alters left–right development in chick and *Xenopus* embryos^{1,8–10}. Although *Shh* expression in the node is necessary and sufficient to induce *Nodal* in the left LPM¹⁰, its exact mechanism is largely unknown. As the action of *Shh* seems to be restricted to cells immediately adjacent to the node, it is difficult to foresee how it could directly activate *Nodal* expression in the left LPM, far away from the node. Tissue explant experiments¹⁰ and the study of laterality defects in conjoined twins¹¹ led to the proposal that *Shh* induces *Nodal* in the LPM through an unknown ('X') secondary signal that is active in the paraxial tissue immediately adjacent to the midline¹⁰.

Here we describe the isolation and characterization of a long-range signal, Caronte (Car), that fulfils the criteria to be such a secondary signal. Car, which is expressed in the left paraxial chick mesoderm, is necessary and sufficient to transmit the *Shh* signal from the node to the left LPM, leading to *Nodal* activation and subsequent establishment of left–right-specific gene expression. Moreover, we provide evidence (by combining *in vivo* misexpression experiments, *in vitro* binding studies, sequence analysis and modelling tools) that Car might activate *Nodal* in the left LPM by relieving a repressive effect of BMPs on *Nodal* transcription, revealing that BMP antagonism is involved in mediating the transfer of left–right positional information from the organizer region to the LPM.

If the initial establishment of asymmetric gene expression in the LPM is essential for proper development, it is equally important to ensure that asymmetry is maintained throughout embryogenesis. An important factor in this regard is Lefty-1, another transforming growth factor- β (TGF- β) family member, that has been proposed to act as a molecular midline barrier that would confine and prevent the 'X' factor from diffusing from the left to the right side of the embryo^{12,13}. Here we show that an excess of Lefty-1 in the left side of the embryo blocks activation of *Nodal* by Car. The opposite experiment, ectopic application of Lefty-1 on the right side, downregulates the expression of the right-sided genes *FGF-8* (ref. 14) (which, in turn, can downregulate Car expression) and *chicken Snail related*¹⁵ (*cSnr*), and upregulates left-sided genes (*Car*, *Nodal* and *Pitx2*). We also show that Car can induce expression of *Lefty-1*. Together, these results uncover a network of molecular interactions that involves a delicate balance of TGF- β activity (including Activin, Lefty-1, BMPs and *Nodal*) and two other secreted factors, FGF-8 and Car, which modulate and restrict the activity of these TGF- β s, allowing proper establishment of the left–right axis.

The molecular interactions described above establish broad domains of side-specific gene expression in the embryo that are translated into specific left–right asymmetric development of organs. *Pitx2* (refs 16, 17; see also ref. 2 and references therein) and *cSnr*¹⁵ are targets of the left–right signalling cascade initiated at gastrulation. We have identified *cNKX3.2*, a new homeobox gene that, like *Pitx2*, may interpret and execute the developmental program dictated by the upstream signalling cascade, so that proper left–right organ asymmetry ensues.

Novel genes involved in left–right development

A novel Cer-like gene, the chick *NKX3.2* gene and the chick *Lefty-1* gene were isolated in a screening designed to identify genes specifically expressed in the left LPM (see Methods). We named the novel Cer-like gene *Caronte* (Car), after the boatman who ferried the souls of the dead across the River Styx in Greek mythology. It encodes a predicted protein of 273 amino acids and contains a hydrophobic signal sequence at the amino terminus and a carboxy-terminal cysteine-knot (CTCK) motif from amino-acids 167 to 244. Sequence comparisons, along with the expression pattern and

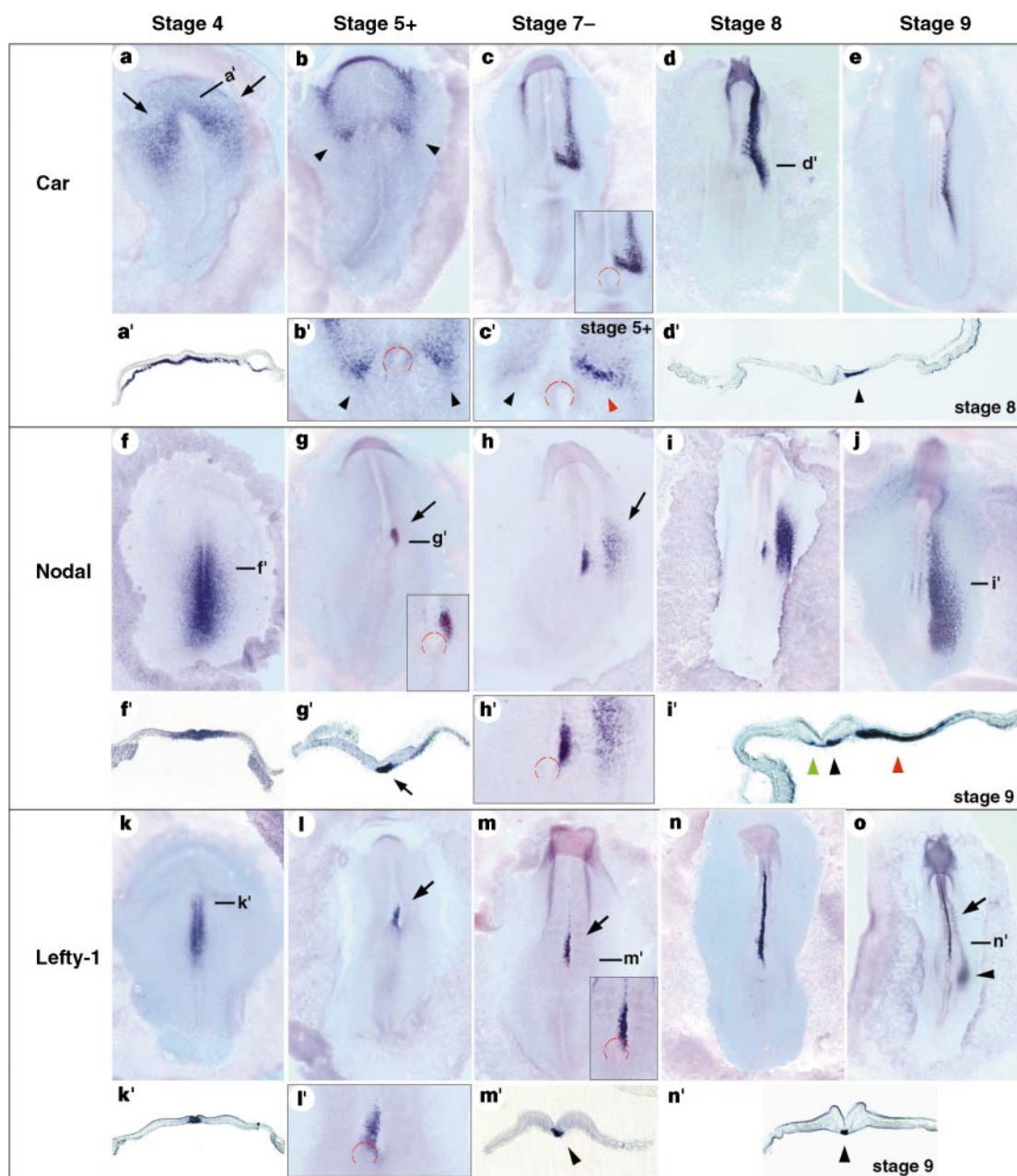


Figure 1 Expression of *Car*, *Nodal* and *Lefty-1* during chick gastrulation. Embryos in all panels are viewed from the ventral side; the left side of the embryo is to the right in all panels unless otherwise indicated. **a**, At stage 4, *Car* is expressed bilaterally in the perinodal region (arrows). **a'**, Cross-section showing expression of *Car* restricted to the mesodermal layer. **b**, Stage 5+ embryo showing symmetrical distribution of *Car* transcripts, with two patches of strong expression flanking Hensen's node (arrowheads). **b'**, Higher magnification of **b**. The node is delineated in red in this and subsequent panels. **c'**, At stages 5+ to 6, the right perinodal expression of *Car* is downregulated. Some embryos at stage 5+ already show strong *Car* expression on the left side (red arrowhead) and almost no expression on the right (black arrowhead). The staining on the left side is adjacent to cells expressing *Nodal* (**g**, inset) and *Lefty-1* (**l**, **l'**). **c**, At stage 7–, *Car* is stronger on the left head mesoderm than on the right (inset, left-specific perinodal expression). **d**, At stage 8, a second domain of *Car* expression appears in the left LPM, extending anteriorly and caudally. **e**, At stage 9, *Car* transcripts start to fade. **d'**, Cross-section of the embryo in **d** showing expression of *Car* in the left LPM (arrowhead). **f**, *Nodal* transcripts are symmetrically expressed in the posterior two-thirds of the primitive streak at stage 4. **f'**, Cross-section of the embryo in **f** showing *Nodal* staining in ectoderm and mesendoderm. **g**, Stage 5+ embryo showing asymmetric expression of *Nodal* in cells

adjacent to the left side of Hensen's node (arrow). Inset, higher magnification of the node. **g'**, Cross-section of the embryo in **g** showing staining of *Nodal* on the left mesodermal layer (arrow). **h**, Stage 7– embryo showing a second domain of *Nodal* expression in the left LPM (arrow). **h'**, Perinodal expression of *Nodal*, still restricted to the left. **i**, Stage 8 embryo showing *Nodal* expression similar to that in **h**. **j**, At stage 9, *Nodal* expands throughout the left LPM (red arrowhead in **i'**) and is expressed not only on the left side of the midline (black arrowhead), but also on the right side (green arrowhead). **k**, At stage 4, *Lefty-1* transcripts are restricted to the anterior half of the primitive streak. **k'**, Cross-section of the embryo in **k** with expression in ectoderm and mesendoderm. **l**, At stage 5+, *Lefty-1* transcripts are expressed asymmetrically in the left side of the node (arrow; see higher magnification in **l'**). **m**, Stage 7– embryo showing *Lefty-1* expression on the left side of the node and the left side of the prechordal mesoderm (arrow; see inset for detail). **m'**, Cross-section of the embryo in **m** depicting expression of *Lefty-1* on the left side of the midline (arrowhead). **n**, Stage 8 embryo. *Lefty-1* midline expression has expanded towards the anterior side of the embryo. **o**, At stage 9, *Lefty-1* expression is symmetrically distributed throughout the notochord (arrow; see also arrowhead in cross-section in **o'**). A patch of *Lefty-1* expression is now detected on the posterior left LPM (arrowhead in **o**).

functional assays described below, indicate that *Car* is a member of the *Cer/Dan* gene family. Other members of this family include *Xenopus Cerberus*¹⁸, *gremlin* (identified in several organisms)¹⁹, and the mouse genes *Dan*^{20,21}, *Cer1* (refs 22–24), *Drm*^{19,25} and *Dante*²⁶. Members of the *Cer/Dan* family of secreted factors are important for patterning the embryo by antagonizing the activities of BMPs and other secreted proteins. The *Car* gene was independently isolated by two other groups^{27,28}.

A second isolated gene fragment translates into a 141-amino-acid sequence that shows homology to Lefty proteins. The full-length gene was obtained by screening a genomic chick library. The predicted amino-acid sequence shows 60% identity to the zebrafish *Antivin*²⁹, 38% identity to mouse *Lefty-1* (refs 30, 31) and 34% identity to mouse *Lefty-2* (ref. 30). Thus, this gene appears to be the chick orthologue of zebrafish *Antivin*. However, based on sequence comparison we cannot tell whether the chick gene is the orthologue of mouse *Lefty-1* or *Lefty-2*. As its pattern of expression closely resembles that of mouse *Lefty-1*, and as *Antivin* and *Lefty-1* are functionally equivalent²⁹, we will call the isolated gene *Lefty-1* hereafter until the putative second *Lefty* is isolated in the chick. Similar nomenclature has been adopted by S. Noji *et al.* (personal communication).

The chick *NKX3.2* gene encodes a 276-amino-acid protein that contains a homeobox from residues 151 to 207 which shows 98% identity with the homeoboxes of the Human *bagpipe*, mouse *NKX3.2*, *Xenopus bagpipe* and *Pleurodeles waltl Nkx3.2* genes. The high degree of conservation of these proteins extends to their C termini. *cNKX3.2* was also independently isolated by another group³².

Car induces *Nodal* in the left LPM

Initially, *Car* transcripts are symmetrically detected in the hypoblast sheet of stage XII chick embryos. As gastrulation proceeds (with the appearance and subsequent elongation of the primitive streak), *Car* transcripts become more abundant, the higher concentration being detected at the anterior end of the mesendoderm layer (data not shown). By stage 4, *Car* is expressed bilaterally in the perinodal region of the embryo, before asymmetric expression of *Shh* or *ActR1IIa* in Hensen's node is detected (Fig. 1a, a'). Subsequently, at stages 5 to 7–, and after the asymmetrical node distribution of *Shh* (in the left) and *ActR1IIa* (in the right) are observed¹, *Car* is downregulated on the right side of the embryo (Fig. 1b, b', c, c'). At stage 7, *Car* is expressed in the left paraxial mesoderm adjacent to the cells expressing *Shh* in the left side of the node (Fig. 1c shows a stage 7–embryo). A second domain of *Car* expression appears in the adjacent left LPM. Whereas the paraxial expression of *Car* disappears at around stage 8 (Fig. 1d), concomitantly with the disappearance of *Shh* expression in the adjacent cells¹⁰, its expression domain in the left LPM expands both anteriorly and posteriorly. At stage 9, *Car* transcripts start to fade away from the left LPM (Fig. 1e), and by stage 10 they are undetectable. As the distribution of *Car* transcripts in the left LPM of stage 6–9 chick embryos appears to precede the onset of *Nodal* expression, we performed parallel whole-mount *in situ* hybridizations for *Nodal* and *Car* genes. *Nodal* transcripts, which are initially symmetrically distributed in the posterior two-thirds of the primitive streak and in immediately adjacent cells (Fig. 1f, f'), become asymmetrically expressed in the mesodermal cells abutting *Shh*-expressing cells in the node at stage 5+ (Fig. 1g, g'). At stage 7–, a second domain of *Nodal* expression appears in the left LPM (Fig. 1h, h'). Subsequent *Nodal* expression seems to follow the expansion of *Car* expression towards the head and anterior and caudal regions of the left LPM¹ (Fig. 1i, j).

The spatio-temporal pattern of *Car* expression indicates that *Car* may be the intermediary paraxial signal that transmits the Shh left–right positional information from the node (Fig. 2a) to the LPM in the chick embryo. Beads soaked in Shh were implanted on the right side of Hensen's node at stage 5, and whole-mount *in situ* hybridi-

zation for *Car* messenger RNA was performed at several timepoints thereafter. Ectopic induction (or maintenance) of *Car* expression was observed on the right LPM (in 18 out of 21 embryos; Fig. 2b). Also, left-sided treatment of stage 5 chick embryos with a blocking antibody that prevents Shh signalling¹⁰ resulted in downregulation of *Car* expression (14/17 embryos; Fig. 2c). As with implantation of beads soaked in Shh, implantation of cell pellets expressing *Car*, or injection of a retroviral vector expressing the *Car* gene (*RCAS–Car*) on the right side of the node at stages 5–7 led to ectopic and broad induction of *Nodal* (58/85 embryos; Fig. 2d) and *Pitx2* (30/62 embryos; Fig. 2f, g), genes that are downstream targets of Shh signalling on the left. Ectopic *Car* expression also caused down-

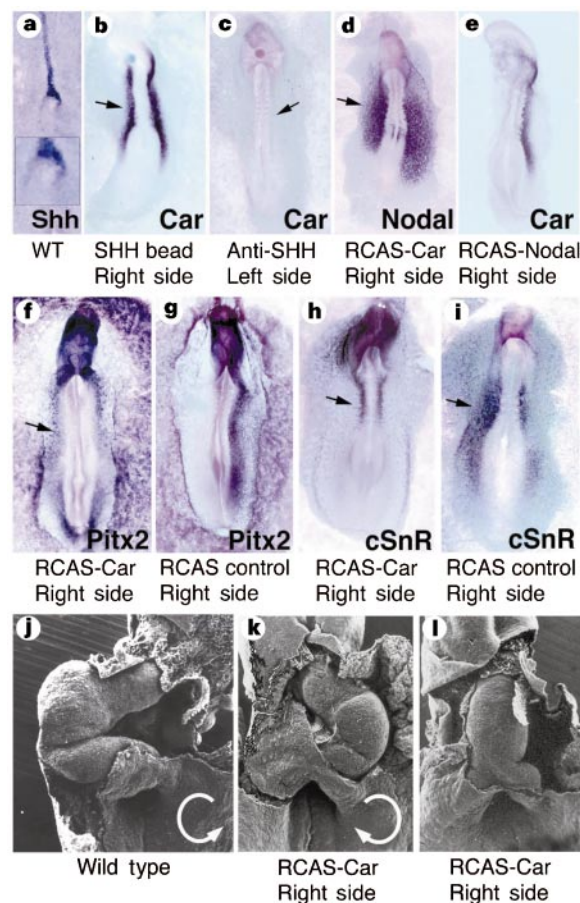


Figure 2 *Car* acts downstream of Shh and upstream of *Nodal* and directs heart looping in the chick embryo. The experimental manipulation and the site of the embryo where it was performed are indicated below each panel. The probes used for *in situ* hybridization are given in each panel. **a**, *Shh* is expressed asymmetrically in the left side of the node at stage 5+; inset, magnification of the node area. Stages of embryos shown in **b–i** are 9–12. **b**, A bead soaked in Shh protein was implanted on the right side of the chick Hensen's node at stage 5. This resulted in ectopic expression of *Car* in the right LPM (arrow). **c**, Implantation of a bead soaked in anti-Shh antibody on the left side of the node of stage 5 chick embryos downregulated the normal expression domain of *Car* in the left LPM (arrow). **d**, Injection of *RCAS–Car* on the right side of Hensen's node at stage 5 resulted in ectopic induction of *Nodal* transcripts (arrow). **e**, Injection of *RCAS–Nodal* on the right side did not affect expression of *Car*. **f, g**, Injection of *RCAS–Car* on the right side of Hensen's node at stage 5 induced ectopic expression of *Pitx2* (arrow in **f**). A control injection using *RCAS–alkaline phosphatase* did not affect *Pitx2* expression in the left LPM (**g**). **h, i**, Expression of *cSnR* in the right LPM (arrow in **i**) was downregulated by injection of *RCAS–Car* on the right side of Hensen's node at stage 5 (arrow in **h**). **j–l**, When embryos injected with the *RCAS–Car* construct on the right side of Hensen's node at stage 5 were allowed to develop until stages 12–13, about 50% of them had reversals of heart looping (**j, k**; white semicircular arrows indicate direction of heart looping). In a few cases the heart was bilaterally symmetric and heart looping did not seem to occur (**l**).

regulation of *cSnr*, a gene specifically expressed in the right LPM (16/24 embryos; Fig. 2h, i). Although misexpression of *Car* on the right side of the embryo induces *Nodal* expression, misexpression of *Nodal* did not induce *Car* expression (0/15 embryos; Fig. 2e). Finally, to ascertain whether the changes in gene expression elicited by ectopic expression of *Car* in the right LPM resulted in left–right morphological alterations, we implanted *Car*-expressing cells on the right side of chick embryos at stages 4–6 and scored for changes in heart morphology at stages 11–13 (Fig. 2j–l). As previously observed after *Shh* or *Nodal* misexpression, heart looping was randomized (9/21 embryos; Fig. 2j, k). In a few embryos, looping was arrested and the hearts appeared to be bilaterally symmetric (3/21 embryos; Fig. 2l). We conclude that expression of *Car* on the left side of the embryo plays an instructive role in determining heart *situs* and looping in the chick embryo.

Our results indicate that *Shh* is necessary and sufficient to induce and/or maintain *Car* expression, which acts upstream of *Nodal*. Together with the fact that proteins encoded by the *Cer*/*Dan* gene family are freely secreted into the extracellular medium^{22,24,26,33}, our data lead us to propose that *Car*, by acting as a long-range signal, fulfils all the requirements to be the factor that mediates the *Shh*-dependent induction of *Nodal* in the left LPM reported in the chick embryo.

Car induces *Nodal* by antagonizing BMPs

Cer-like secreted factors can antagonize BMP activity by binding directly to BMPs, thus blocking their interaction with BMP

receptors^{19,26,33}. Sequence analysis of the Cer-like protein *Car* shows that it contains the structural domain that mediates the interaction of Cer proteins with BMPs³³ (see below). Several BMPs are expressed in the LPM in bilaterally symmetrical patterns at the time at which *Nodal* expression appears in the left LPM^{34–36} (for example, Fig. 3a shows a composite of *BMP-2* and *BMP-4* patterns). Around stage 7, *Nodal* expression is stronger in a subdomain that also expresses very high levels of *Car* (red asterisks in Fig. 3b, c). A more lateral subdomain of weaker *Nodal* expression abuts the region where *Car* transcripts are also more faintly expressed (green asterisks). Finally, *Nodal* is not detected in even more lateral regions where *BMP* transcripts (Fig. 3a) are present but *Car* is completely absent (yellow asterisks in Fig. 3a–c). As expected, after we implanted *Car*-expressing cells on the right side of the embryo, *Nodal* was induced in a high percentage of embryos (58/85 embryos; Fig. 3d, arrow). However, *Nodal* induction is observed much less frequently when a bead soaked in BMP is placed next to the *Car*-producing cells (only 3/34 embryos showed *Nodal* induction; Fig. 3e). Moreover, when a bead soaked in BMP is applied on the left side of the node, *Nodal* expression is downregulated (10/30 embryos; Fig. 3f, arrow). This indicates that *Car* and BMP have antagonistic effects on *Nodal* expression, and that *Car* might activate and/or maintain *Nodal* expression in the left LPM by antagonizing a repressive effect of BMP.

To validate this hypothesis independently *in vivo*, we used *Noggin*, a secreted factor structurally unrelated to *Car* that can bind BMPs³⁷ and antagonize their activities^{37–39}. We misexpressed *Noggin* on the right side of the node at stages 4–5 either by implanting a pellet of *Noggin*-expressing cells or by infecting embryos with *RCAS-Noggin*³⁸. This resulted in induction of *Nodal* expression (Fig. 3g). We confirmed that *Car* itself may act as a BMP antagonist by performing two different assays in chick embryos, where antagonism of BMP activity is well characterized (see Supplementary Information). First, *Car* can abolish formation of cartilage nodules in micromass cultures of limb mesenchymal cells, and this effect can be rescued by adding BMP protein. Second, *Car* misexpression in chick limbs has the same effect as *Noggin* misexpression³⁸.

Interaction of *Car* with BMPs and *Nodal*

To test for a possible direct interaction between *Car* and BMPs, we produced in COS7 cells a triple myc-epitope-tagged version of *Car* (*Car-myc3*), and tested by immunoprecipitation its possible interactions with several members of the TGF- β superfamily, including *BMP-4*, *BMP-5*, *Nodal*, *GDF-5* and *Activin* (Fig. 4a; see Methods). When COS7-conditioned medium containing *Car-myc3* was incubated with *BMP-4*, immunoprecipitation with an anti-myc antibody pulled down *BMP-4*, as detected by western blotting using an antibody against *BMP-4*. Similarly, when conditioned medium containing *Car-myc3* was incubated with ³⁵S-*Nodal*, immunoprecipitation with an anti-myc antibody pulled down ³⁵S-*Nodal*. When similar experiments were performed with *BMP-5*, a tagged version of *GDF-5* (*GDF-5-Flag*) or *Activin*, these proteins were not pulled down by immunoprecipitation with the anti-myc antibody. We conclude that *Car* interacts specifically with certain members of the TGF- β superfamily (*BMP-4* and *Nodal*). Similar results have been obtained for the *Xenopus* Cer protein, which also binds *BMP-4* and *Nodal* but not *Activin*³³. In our experimental setting, the *Car*–*Nodal* interaction is reduced by about 50% when human Cer is included in the binding reaction (Fig. 4a).

We also performed sequence analysis and modelling to gain insight into the structural basis for the antagonistic interaction between Cer-like and BMP-like proteins. A multiple sequence alignment (MSA) with CLUSTALX⁴⁰ of the cysteine-rich region of several Cer-like proteins, together with BMPs and Nodals, reveals two distinct groups with different sequence patterns (Fig. 4b). A Cer-like group, that we shall call β -like, is characterized by a C-

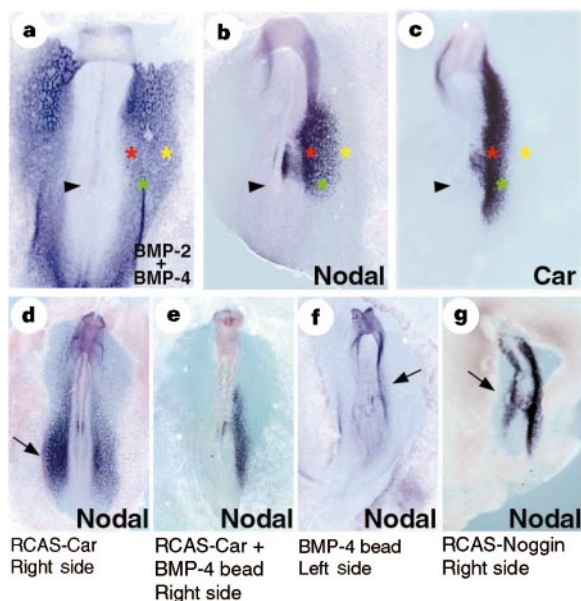


Figure 3 Antagonism of BMPs by *Car* regulates *Nodal* expression. **a**, The sum of the expression patterns of *BMP-2* and *BMP-4* in a stage 7 embryo reveals that BMPs are expressed in broad symmetrical domains in the left and right LPM. **b, c**, *Nodal* expression in the LPM at the same stage is restricted to the left side, where *Car* (**c**) is also expressed. *Nodal* expression is stronger in the region that also expresses very high levels of *Car* (red asterisks in **a–c**). Green asterisks mark a more lateral subdomain of weaker *Nodal* expression (**b**) that overlaps the region where *Car* transcripts are also more weakly expressed (**c**). *Nodal* is absent from the regions indicated by yellow asterisks, where BMPs are present but *Car* is completely absent. Arrowheads in **a–c** point to the node. **d**, When *Car*-expressing cells (*RCAS-Car*) were implanted on the right side of Hensen's node at stage 5, *Nodal* was ectopically induced (arrow). **e**, Induction of *Nodal* was much less frequent when a bead soaked in *BMP-4* (1 mg ml^{−1}) was implanted with a graft of *Car*-expressing cells (**e** shows an embryo where induction of *Nodal* by *Car* on the right side has been completely inhibited). **f**, When a bead soaked in *BMP-4* (1 mg ml^{−1}) is applied on the left side of the node at stage 4, *Nodal* expression in the left LPM is strongly downregulated (arrow). **g**, When cells expressing the BMP antagonist *Noggin* are implanted on the right side of the node at stage 5, *Nodal* is ectopically induced (arrow).

terminal cysteine knot, and has two cysteine residues at positions 15 and 83, together with a short deletion in positions 41–44 and 57–64, as hallmarks (Fig. 4b). BMP-like proteins, which we shall describe as α -like, show a TGF- β 2-like pattern with a characteristic α -helix between positions 48–54 (Fig. 4b).

A survey of the structural classification of proteins (SCOP) database reveals an example of an interaction between a β -like and an α -like sequence: the heterodimeric (α and β) structure of human chorionic gonadotropin. This indicates that the interaction

between Cer-like and BMP-like proteins could be reminiscent of the gonadotropin mode of interaction. This structural inference is supported by sequence analysis. After incorporation of the human gonadotropin sequences in the MSA of Cer-like and BMP-like sequences, followed by further clustering, we found that the β -chain is more similar to Cer-like sequences than to the α -chain, which is more similar to BMP-like sequences (Fig. 4b). The available information, both from the sequence analysis and the structural model, indicates a mode of interaction between BMP-like and Cer-

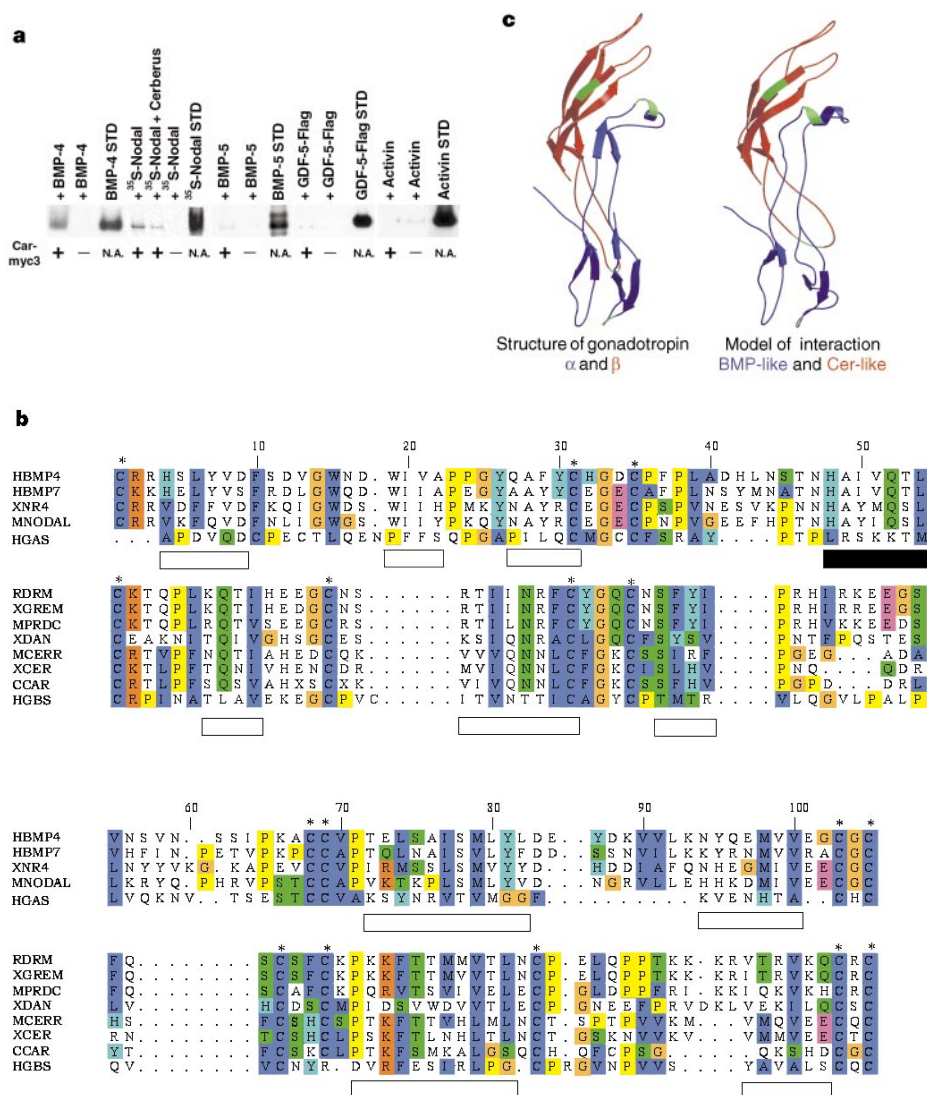


Figure 4 Interaction of Car with BMPs: biochemical evidence and structural model. **a**, COS7-conditioned medium containing Car–myc3 was incubated with several TGF- β proteins (see Methods), and an anti-myc antibody was used to immunoprecipitate Car–myc3 and associated proteins, which were then detected either by phosphoimaging (35 S–m Nodal) or by western blotting using antibodies against the corresponding proteins or their epitope tags. Car–myc3 binds to BMP-4 and Nodal but not to other TGF- β s such as BMP-5, GDF-5 and Activin. Human Cerberus competes with Car–myc3 for binding to Nodal (**a**). The lanes marked STD refer to independent assays performed with the same batches of proteins used for the experiments involving Car–myc3, which confirm that all the proteins used were biochemically active in the immunoprecipitation assay. N.A., not applicable: those independent assays do not involve Car–myc3 (details available upon request). **b**, MSA of selected cysteine-rich regions of the BMP-like proteins human BMP-4 (HBMP4), human BMP-7 (HBMP7), *Xenopus* Nodal related (XNR4) and mouse Nodal (MNODAL), aligned to the cysteine-rich region of the human gonadotropin α -subunit (HGAS); and of cysteine-rich regions of the Cer-like proteins rat-DRM (RDRM), *Xenopus* Gremlin (XGREM), mouse PRDC (MPRDC), *Xenopus* Dan (XDAN), mouse Cer-related (MCERR), *Xenopus* Cerberus (XCER) and chicken Caronte (CCAR), aligned to the cysteine-

rich region of the human Gonadotropin β subunit (HGBS). The MSA was made using ClustalX⁴⁰. Colours indicate conserved properties. Consensus secondary structure prediction is depicted by open (β -strand) or filled (α -helix) rectangles below the alignments. Asterisks denote conserved cysteine residues inside each group. **c**, Model of interaction between BMP-like and Cer-like cysteine-rich regions. Left, ribbon diagram of the structure of the α - (blue) and β -chains (red) of the human gonadotropin hormone. Right, model by homology using MODELLER (details available upon request) of the cysteine-rich region of human BMP-4 (blue) and chicken Car (red). Green, key residues unmasked by a Factor Analysis (FA). In both cases, they cluster around the α -helix and the loop between the last two β strands in the blue chains and around the second β -like gonadotropin sequences. A similar situation is observed for the BMP-like sequences, where the residues detected are equivalent to those observed if the α -like gonadotropin sequences are used. Mapping these residues, respectively, onto the structure of the α - and β -chains of the human gonadotropin (left) or onto a homology model of the interaction between BMP-like and Cer-like sequences based on the previous structure (right), shows that they appear at the interface between chains that correspond to the functional areas of the complex.

like proteins similar to that observed between the α - and β -chains of the human gonadotropin (Fig. 4c).

As Car seems to be a multifunctional antagonist, able to bind BMP and Nodal proteins, the question arises of why the presence of Car in the left LPM does not interfere with Nodal activity. As a result of Car antagonizing BMP activity in the LPM, *Nodal* transcripts begin to accumulate quickly at stage 7 and reach high levels throughout the left LPM at a time at which an accumulation of Nodal protein can presumably overcome the antagonism by Car. Thus it is unlikely that Car significantly interferes with the activation of Nodal transcrip-

tional targets (such as *Pitx2*) at that stage of development. Consistent with this interpretation, when we deliver Car to the left side of the embryo by implanting *Car*-producing cell grafts, *Nodal* transcription is strongly upregulated, even outside its normal domain of expression (data not shown).

Regulation of *Car* expression by FGF-8

Downregulation of *Car* expression on the right side of the embryo is essential for normal left–right development, as ectopic presence of *Car* on the right side results in laterality defects. A good candidate for repressing *Car* on the right side of the embryo is FGF-8. The *FGF-8* gene begins to be expressed asymmetrically in the node under the control of Activin βB^{14} around the time when *Car* is downregulated on the right side of the embryo. Moreover, ectopic application of FGF-8 protein inhibits the expression of the left-specific genes *Nodal* and *Pitx2*, and induces expression of the right-specific gene *cSnR* when applied to the left side of the embryo¹⁴. Beads soaked in FGF-8 implanted in the left side of the node at stage 5 downregulate *Car* transcripts (8/20 embryos; Fig. 5a, b). To further substantiate the role of FGF-8 as a repressor of *Car*, we inhibited FGF signalling in two ways. First, when beads soaked in the FGF receptor-1 (FGFR-1) inhibitor SU5402 (ref. 41) were applied to the right side of the node, *Car* expression was maintained and/or induced (5/20 embryos; Fig. 5c, d), and *Nodal* was ectopically expressed on the right side (6/22 embryos; Fig. 5e, f). The embryo in 5f is older than that in 5e). Second, we repeated the experiment using beads soaked in a soluble version of a truncated Activin receptor (ActRII-ECD) that specifically inhibits Activin signalling¹⁶. This resulted in the downregulation of *FGF-8* expression on the right posterior side of the node (9/15 embryos; red arrow in Fig. 5g, compare with 5h) and maintenance and/or induction of *Car* expression on the right LPM (9/19 embryos; Fig. 5i). Conversely, implantation of Activin on the left side of the embryo led to a downregulation of *Car* expression in the left LPM (12/18 embryos; Fig. 5j, k).

Our results are compatible with *FGF-8* expression on the right side of the node acting as a negative regulator of *Car* expression. Thus, *Car* is activated (or maintained) by *Shh* in the left side of the embryo and repressed by *FGF-8* in the right side. This ensures that by stage 6 *Car* transcription is restricted to the left side of the gastrulating chick embryo.

Lefty-1 stabilizes asymmetric gene expression

The *Lefty-1* gene has been proposed to be vital for restricting the expression of *Nodal*, *Lefty-2* and *Pitx2* to the left side of the embryo, acting as (or inducing) a ‘midline barrier’ that would prevent the presence of the ‘X’ factor on the right side of the embryo^{12,13}. We have investigated the role of *Lefty-1* in left–right determination in the chick, and its possible interaction with *Car*.

Lefty-1 appears earlier in the chick than in the mouse embryo. In the mouse, *Lefty-1* is expressed predominantly in the presumptive floorplate, beginning at the two-somite-pair stage³⁰. In the chick, *Lefty-1* transcripts are detected in Koller’s sickle at the posterior margin of the chick blastoderm at stages X–XII (data not shown) in cells that will later contribute to Hensen’s node, the chick organizer⁴². Subsequently, as the primitive streak elongates, *Lefty-1* transcripts become restricted to the anterior half of the streak (Fig. 1k, k’). At stage 5+, *Lefty-1* transcripts are observed in a pattern overlapping the domain of *Shh* expression on the left side of the node (Fig. 1l, l’, compare with *Shh* in Fig. 2a). From stages 6 to 8, *Lefty-1* expression is restricted to the left side of the prechordal mesoderm, and by stage 8, coinciding with the disappearance of the left-sided *Shh* expression, *Lefty-1* becomes symmetrically distributed throughout the caudal notochord (Fig. 1n; 1n’ is a section of the embryo shown in 1o). Before *Lefty-1* is no longer detectable at stage 11, a small domain of expression is observed in the left posterior LPM overlapping the *Nodal*-expressing cells (Fig. 1o, compare with 1j).

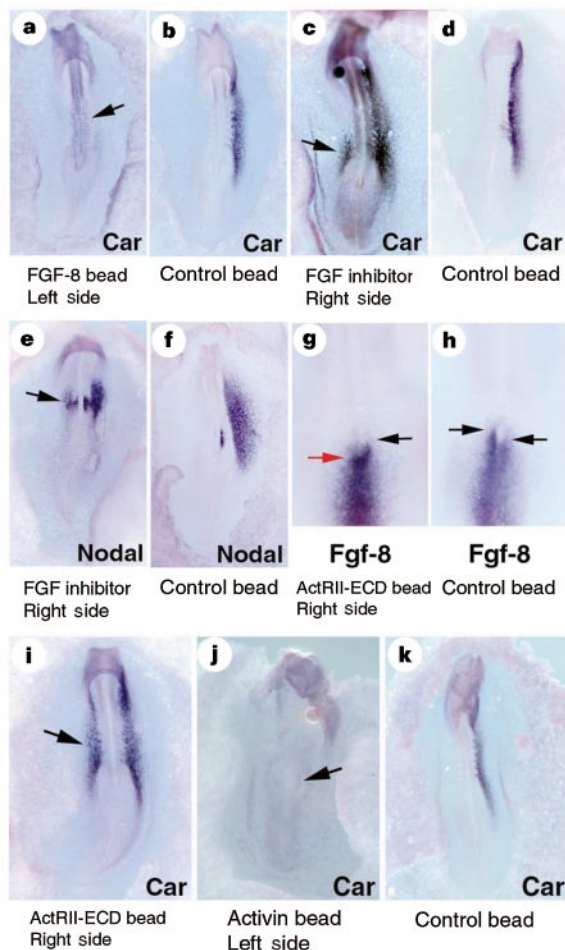


Figure 5 FGF-8 and Activin regulate *Car* expression. The embryos shown in **a–d** and **i–k** are stages 7–9. **a**, When a bead soaked in FGF-8 protein (1 mg ml^{-1}) was implanted on the left side of Hensen’s node at stage 5, *Car* transcripts were downregulated (arrow). **b**, *Car* expression was not affected by control BSA-soaked beads. **c**, Inhibition of FGF signalling by application of SU5402 (1 mg ml^{-1}) to the right side of Hensen’s node at stage 5 maintained and/or induced *Car* expression (arrow). **d**, Normal *Car* expression when BSA control beads were applied. **e**, A bead soaked in SU5402 implanted on the right side of Hensen’s node at stage 5 induced ectopic expression of *Nodal* on the right side (arrow). **f**, Control beads had no effect on *Nodal* expression (slightly older embryo). **g**, Stage 6 embryo. During normal development, *FGF-8* is expressed in the right but not left posterior portion of the node. When a bead soaked in the truncated Activin receptor (ActRII-ECD) (0.5 mg ml^{-1}) was applied to the right side of Hensen’s node at stage 4, the right-sided *FGF-8* expression in the node was downregulated (red arrow). Black arrow, normal anterior level of *FGF-8* on the left side of the node. **h**, Stage 6 embryo. Control beads had no effect on normal expression of *FGF-8* in the right posterior portion of the node (arrows). **i**, A bead soaked in ActRII-ECD (0.5 mg ml^{-1}) applied to the right side of the node at stage 4 maintained and/or induced *Car* expression on the right side (arrow). **j**, Application of a bead soaked in Activin (0.5 mg ml^{-1}) on the left side of the node at stage 4 downregulated expression of *Car* in the left LPM (arrow). **k**, BSA control beads did not affect *Car* expression.

When either cells expressing mouse *Lefty-1* or an RCAS virus containing the mouse *Lefty-1* gene were applied at stages 4–5 to the right side of the node, we observed repression of both *FGF-8* (8/15 embryos; Fig. 6a, b) and *cSnR* (8/20 embryos; Fig. 6f), and activation of left-specific genes such as *Car* (8/15 embryos; Fig. 6c), *Nodal* (9/18 embryos; Fig. 6d) and *Pitx2* (10/25 embryos; Fig. 6e). These results, together with those shown in Fig. 5, lead us to propose that *Lefty-1* might be able to antagonize Activin. Several plausible mechanisms can be envisioned, including competitive binding of *Lefty-1* to the Activin receptor, which would result in an inactive

ligand–receptor complex that would not be able to induce *FGF-8* on the right side of the node. A similar role in inactivating Activin pathways has been proposed for Antivin, whose function in zebrafish can be substituted by mouse *Lefty-1* (ref. 29). These results indicate that the early domain of expression of *Lefty-1* in the left side of the embryo (adjacent to the node) is important as a safety mechanism that ensures that the Activin pathway is completely inactive on the left side of the embryo, allowing continued expression of left-specific genes such as *Car*, *Nodal* and *Pitx2*.

An excess of mouse *Lefty-1* on the left side of the chick node blocks activation of *Nodal* expression in the left LPM¹³. Here we show that, although expression of *Nodal* is either absent or down-regulated after application of mouse *Lefty-1* on the left side of the stage-5 chick node (9/15 embryos; Fig. 6h), *Car* expression is not affected (0/15 embryos; Fig. 6g). As we have shown that *Car* is necessary and sufficient to activate *Nodal*, these results indicate that *Lefty-1* may antagonize *Car* activity without affecting its transcription. One possibility is that *Lefty-1* could antagonize *Car* activity by binding to it. In doing so, *Lefty-1* would limit *Car* diffusion and would effectively interfere with its function. This assumption goes along with the hypothesis that the expression of *Lefty-1* on the left side of the node and on the left side of the midline could serve as a barrier to prevent spreading of *Car* (or, in general terms, the *Shh*-

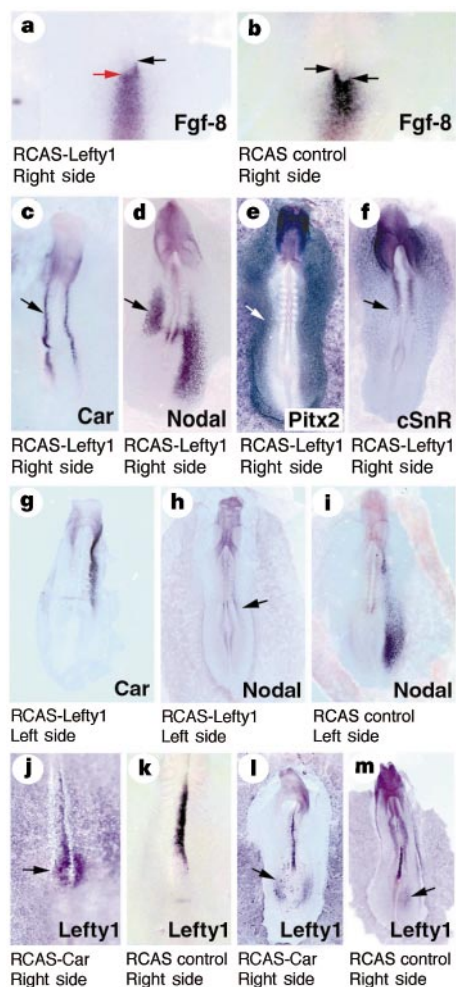


Figure 6 Interaction between *Lefty-1* and *Car* during the establishment of left–right asymmetry. **a**, Stage 6 embryo. When cells expressing mouse *Lefty-1* (RCAS-*Lefty-1*) were implanted on the right side of Hensen's node at stage 4, the right-sided *FGF-8* expression in the node was downregulated (red arrow). Black arrow, normal anterior *FGF-8* expression on the left side of the node. **b**, Stage 6 embryo. Control cells expressing alkaline phosphatase had no effect on the expression of *FGF-8* in the right posterior portion of the node (arrows). Embryos in **c–i** are at stages 8–10. **c–e**, A retrovirus vector containing the mouse *Lefty-1* gene was injected at stage 4 to the right side of the node, producing ectopic induction of *Car* (arrow in **c**), *Nodal* (arrow in **d**) and *Pitx2* (arrow in **e**). **f**, The normal expression domain of *cSnR* in the right LPM was downregulated by injection of RCAS-*Lefty-1* at stage 4 on the right side of the node (arrow). **g**, RCAS-*Lefty-1* on the left side of Hensen's node at stage 4 did not affect the normal expression of *Car* in the left LPM. **h**, The same experiment downregulated *Nodal* expression (arrow). **i**, The control virus (RCAS-alkaline phosphatase) had no effect on *Nodal* expression. **j–m**, When a graft of *Car*-expressing cells (indicated as RCAS-*Car*) was implanted on the right side of Hensen's node at stage 4, *Lefty-1* expression was upregulated on the right side of the node (arrow in **j**; compare with control in **k**). Infection with RCAS-*Car* produced upregulation of *Lefty-1* on the posterior side of the right LPM (arrow in **l**; compare with the control experiment in **m**, where normal expression in the left LPM is indicated by an arrow).

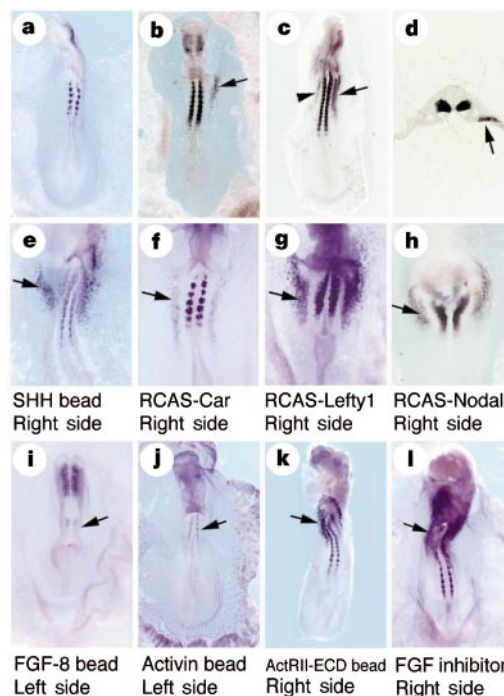


Figure 7 *cNKK3.2* is a novel target of the left–right signalling pathway. **a**, Expression of the *cNKK3.2* gene in somites at stage 8. **b**, At stage 10, transcripts are still detected in the somites and two new domains of expression are observed in the head mesoderm and the left LPM (arrow). **c**, As well as the head mesoderm, somites and left LPM (arrow), a faint expression domain is now observed in the right LPM (arrowhead). **d**, Cross-section (at the level of the arrow) of the embryo shown in **b** displaying symmetrical expression of *cNKK3.2* in the somites and left-specific staining in the LPM (arrow). **e–h**, Application of beads soaked in Shh protein (**e**) and RCAS-*Car* (**f**), RCAS-*Lefty-1* (**g**) or RCAS-*Nodal* (**h**) viruses to the right side of the Hensen's node at stage 5 induced ectopic expression of *cNKK3.2* in the right LPM (arrows). **i**, Implantation of a bead soaked in FGF-8 (1 mg ml⁻¹) on the left side of Hensen's node at stage 5 downregulated expression of *cNKK3.2* in the left LPM and somites (arrow). **j**, Application of a bead soaked in Activin (1 mg ml⁻¹) on the left side of Hensen's node at stage 5 downregulated expression of *cNKK3.2* in the left LPM and somites (arrow). **k**, Application of the truncated activin receptor ActRII-ECD (0.5 mg ml⁻¹) to the right side of Hensen's node at stage 5 enhanced expression of *cNKK3.2* in the right LPM (arrow). **l**, A similar result (arrow) was obtained when the FGFR-1 inhibitor SU5402 (1 mg ml⁻¹) was applied to the right side of the embryo.

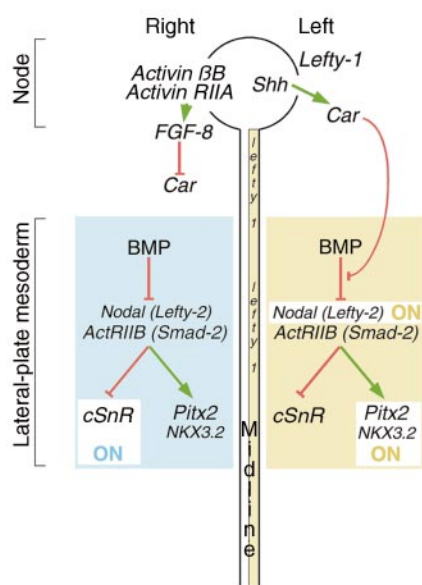


Figure 8 Role of *Car* in the genetic cascade that determines left–right development. Control of *Car* expression by *Shh* and FGF-8, together with a possible barrier mechanism elicited by Lefty-1, restricts *Car* activity to the left side of the embryo, where it can antagonize the repressive effect of BMPs on *Nodal* transcription. *Nodal* activates downstream left-specific genes and represses right-specific genes in the LPM. Green arrows indicate activation and red lines indicate repression; in the case of *Car* the red line indicates antagonism of BMPs by direct binding to them. It cannot be ruled out that a factor other than *Car* might actually be the ‘X’ factor that diffuses to the right (or is transcribed on the right) in the absence of *Lefty-1*, according to the barrier model¹⁸. However, as *Car* fulfils all the required criteria to be the ‘X’ factor, even if that additional factor exists, it probably acts through *Caror* in a pathway related to *Car*. A better understanding of the role of the *Car* gene in left–right development must await its identification and ablation in the mouse. This is a simplified model intended to illustrate the role of *Car*, and only some of the genes involved in left–right development are included.

dependent ‘X’ factor) from the left to the right side of the embryo. Alternatively, Lefty-1 might repress *Nodal* transcription by mechanisms unrelated to *Car*. An added twist is that Lefty proteins seem to be able to antagonize *Nodal* activity by mechanisms that could involve either competition with *Nodal* for a common receptor, formation of inactive heterodimers with *Nodal* or both. Identification of the murine *Car* and analysis of its pattern of expression in wild-type and *Lefty-1*^{−/−} mice will be necessary to determine the nature of the regulatory relationship between *Car* and *Lefty-1*. *Car*-expressing cells implanted on the right side of the embryo can induce expression of *Lefty-1* on the right side of the midline (8/15 embryos; Fig. 6j, k) and in the right LPM (7/15 embryos; Fig. 6l, m). As *Car* presumably acts as an extracellular antagonist, it might induce *Lefty-1* in the midline by antagonizing a repressive activity of some TGF-β member on *Lefty-1* transcription. BMPs are expressed along the primitive streak and are likely candidates to encode this repressive activity. Of course, additional regulators may control *Lefty-1* expression, as even ectopic expression induced by *Car* is similar to the endogenous pattern observed on the left side of the embryo.

cNKX3.2 is a target of the left–right pathway

Finally, we have identified the homeobox gene *cNKX3.2*, which is the chick homologue of mouse *BapX1/NKX3.2* (ref. 43); the same gene was independently isolated by another group³². *NKX* genes are involved in cellular differentiation and organogenesis in different organisms^{44,45}. *cNKX3.2* begins to be expressed at stages 8–9, where it is observed in the somites (Fig. 7a). At stages 10–11, *cNKX3.2* appears in the head mesoderm and in the left LPM (Fig. 7b, d). Around stage 14, faint expression is also seen in the right LPM (Fig. 7c). At later stages *cNKX3.2* is expressed in other embryonic structures, including

gut and limbs (data not shown), with a very similar pattern to that reported for its mouse and *Xenopus* orthologues.

We decided to investigate in detail the mechanisms that regulate *cNKX3.2* transcription, in order to place it in the genetic cascade that controls left–right asymmetry. Misexpression of either *Shh* (16/18 embryos; Fig. 7e), *Car* (19/28 embryos; Fig. 7f), mouse *Lefty-1* (12/28 embryos; Fig. 7g) or *Nodal* (14/30 embryos; Fig. 7h) in the right side of the embryo all induce strong *cNKX3.2* expression in the right LPM. Conversely, misexpression of either FGF-8 (11/18 embryos; Fig. 7i) or *Activin* (8/17 embryos; Fig. 7j) in the left side of the embryo inhibits the normal domain of *cNKX3.2* expression. We confirmed the specificity of the effect of these two right signals by misexpressing either a soluble version of a truncated *Activin* receptor (which inhibits *activin* signalling; 8/23 embryo; Fig. 7k) or the specific FGFR-1 inhibitor SU5402 (6/19 embryos; Fig. 7l) on the right side of the embryo. Both manipulations induce *cNKX3.2* expression in the right LPM. In general, *cNKX3.2* responds to upstream left–right signals in a similar way to *Pitx2*. Like *Pitx2*, we consider *cNKX3.2* to be a target of the early patterning network of left–right signals involved in executing the cellular changes that are responsible for organ morphogenesis.

It is debatable whether a general mechanism of left–right determination (Fig. 8) applies to all vertebrates. Although the left-specific expression of important determinants such as *Nodal* and *Pitx2* seems to be conserved in all vertebrates examined so far, this may not be the case for upstream regulators. For example, expression of *Shh* and FGF-8 has not been reported to be asymmetric in *Xenopus* or mouse. However, *Shh*^{−/−} and *FGF-8*^{−/−} mutants show laterality defects, including altered expression of *Nodal*, *Pitx2* and *Lefty-2* (refs 46–48). In the mouse, *Shh* seems to be required to induce and/or maintain the midline barrier that has been proposed to restrict expression of *Nodal*, *Lefty-2* and *Pitx2* to the left side of the embryo^{12,13}. In this model, *Shh* would not be required for left-sided expression of these genes. This interpretation contrasts with its proposed role as a left determinant in the chick. A more accurate assessment of the degree of evolutionary conservation of left–right determination mechanisms awaits a future analysis of the roles of *Shh*, FGF-8 and other genes involved in left–right development in different organisms; thus extending the analysis of the network of molecular interactions described here to other vertebrates should provide further insight into the general mechanisms of left–right determination. □

Methods

Cloning of *Car*, *Lefty-1* and *cNKX3.2*

Left and right LPM mRNAs from embryos at stages 6–12 were isolated using standard protocols. A left LPM subtracted complementary DNA library was obtained using the Clontech PCR-Select cDNA subtraction kit. Seventy clones were selected at random and used to generate digoxigenin-labelled probes. After confirming that three of the clones represented differentially expressed genes, we sequenced them in both strands. The *Lefty-1* fragment was isolated indirectly from the PCR-Select differential screening and used to screen a chick genomic library to obtain the full-length *Lefty-1* gene using standard protocols. The *Car* and *cNKX3.2* full-length clones were obtained by screening stage 5 and 12 chick cDNA libraries, respectively, with noncoding fragments isolated from the PCR-Select differential screening (for *Car*) and by using a 5′ RACE-Smart kit from Clontech (for *cNKX3.2*). The chick *Car*, *cNKX3.2* and *Lefty-1* sequences have been deposited in GenBank under accession numbers AF179484, AF179482 and AF179483, respectively.

Retroviral infection and bead implantation

Chick embryos were explanted and grown *in vitro* as described⁴⁹. RCAS (subtype A) retroviral stocks containing full-length *Car*, *Nodal*, mouse *Lefty-1* and *alkaline phosphatase* (used as control) were produced as described¹⁶. Embryos were either infected with RCAS viruses by air pressure and/or grafted with RCAS-infected cells on either side of the chick blastoderm near Hensen’s node. Beads were soaked in *Shh* protein, anti-*Shh* antibody and FGF as described¹⁰. The FGFR1 inhibitor SU5402 (ref. 41; CalBiochem) was used at 1 mg ml^{−1}.

In situ hybridization

After viral infection and/or bead implantation, we processed embryos for whole-mount *in situ* hybridization as described⁴². Sectioning of stained embryos was as described¹⁶.

Antisense probes for *Car* and *cNKX3.2* spanned the entire open reading frame (ORF). For *Lefty-1* we used the entire isolated fragment. We generated a 450-base-pair (bp) probe corresponding to the 3' untranslated region that gave an identical pattern of expression to that obtained with the cDNA fragment (data not shown). The rest of the probes were produced as described¹⁶.

Co-immunoprecipitation experiments

A triple myc-tagged version of *Car* (Car-myc3) was obtained by cloning a *Car* insert into the mammalian expression vector pMT21-myc3, bringing the *Car* ORF without a stop codon in frame with a triple myc-tag (5'-GAG CAG AAG CTG ATA TCC GAA GAA GAC CTC GGC GGA GAG CAG AAG CTC ATA AGT GAG GAA GAC TTG GGC GGA GAG CAG AAG CTT ATA TCC GAA GAA GAT CTC GGA CCG TGA TAA-3'). The construct was verified by restriction analysis and dideoxy sequencing. Conditioned media from COS7 cells transfected with the pMT21-Car-myc3 construct was obtained using standard protocols. Briefly, serum-free conditioned media were collected two days after the start of transfection, cleared of cell debris and made up to 5 mM EDTA. Conditioned media were stored at 4°C. The expression of Car-myc3 was verified by western blotting for the myc epitope. Under nonreducing conditions, Car-myc3 had a relative molecular mass of ~40,000, consistent with the predicted molecular mass of Car-myc3 and accounting for glycosylation at two potential N-linked glycosylation sites. Dimeric and multimeric forms of Car-myc3 were also observed, as seen for other members of the Cer/Dan family of BMP antagonists (A. N. Economides and N. Stahl, unpublished results). A similar procedure was used to obtain conditioned medium containing human GDF-5-Flag⁵⁰. Car-myc3 (1 ml of COS7-derived serum-free conditioned medium) was incubated with human BMP-4 (0.5 µg ml⁻¹; R&D Systems), human BMP-5 (0.5 µg ml⁻¹; R&D Systems), human GDF-5-Flag (0.2 ml COS7-derived conditioned medium) or mouse Nodal (mBMP-16; provided as ³⁵S-mNodal expressed in *Xenopus laevis* oocytes). The formation of a stable complex between Car-myc3 and these different TGF-βs was determined by immunoprecipitating Car-myc3 and associated proteins using an anti-myc monoclonal antibody (9E10; 1 µg ml⁻¹) bound to Protein A-Ultralink (Pierce). The binding reaction was carried out in serum-free conditioned medium after it was made 20 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20 (TBST), by addition of a 10× concentrate of these reagents. Binding was allowed to proceed for 1 h at 25°C in a reaction volume of 1.1 ml, with continuous mixing to keep the Protein A-Ultralink (20 µl bead volume) in suspension, after which point the beads were spun down, washed once with TBST, transferred to new eppendorf tubes and washed three more items with TBST. Proteins bound to the beads were solubilized by addition of 25 µl of Laemmli SDS-PAGE sample buffer and loaded onto 4–12% NuPAGE/MES gradient gels (Novex), which were run under reducing conditions. The proteins were subsequently transferred onto Immobilon P and western blotted to detect human BMP-4, human BMP-5 or human Activin using antisera raised against the respective proteins (R&D Systems). In the case of human GDF-5-Flag, we used a monoclonal antibody raised against the Flag tag (M2; Kodak). ³⁵S-mNodal was visualized using a Phosphorimager (Fuji). None of the TGF-β proteins tested binds to the beads when Car-myc3 is excluded from the reaction mixture and instead substituted with COS7-derived conditioned medium from cells that had been transfected with a pMT21 plasmid.

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Evidence for a positive cosmological constant from flows of galaxies and distant supernovae

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Recent observations^{1,2} of high-redshift supernovae seem to suggest that the global geometry of the Universe may be affected by a ‘cosmological constant’, which acts to accelerate the expansion rate with time. But these data by themselves still permit an open universe of low mass density and no cosmological constant. Here we derive an independent constraint on the lower bound to the mass density, based on deviations of galaxy velocities from a smooth universal expansion^{3–7}. This constraint rules out a low-density open universe with a vanishing cosmological constant, and together the two favour a nearly flat universe in which the contributions from mass density and the cosmological constant are comparable. This type of universe, however, seems to require a degree of fine tuning of the initial conditions that is in apparent conflict with ‘common wisdom’.

The standard cosmological model based on Einstein’s gravity and global homogeneity is characterized by two fundamental parameters that measure the main contributions to the total energy density (in terms of a ‘critical’ density): the mean mass density Ω_m and the cosmological constant Ω_Λ . The latter, an intrinsic part of the theory of general relativity, represents a uniform energy density that is associated with the vacuum (not the mass), and, if positive, acts as a repulsive component of the global gravitational force field. The values of these parameters determine the type of the universe we live in, its global extent and ultimate fate, as follows. The parameter plane (Fig. 1) is divided by three lines into six regions. The line $\Omega_m + \Omega_\Lambda = 1$ defines a ‘flat’ euclidean geometry, favoured by theories of inflation in the early universe; it separates curved-space (non-euclidean) models of ‘closed’ finite extent (above) and ‘open’ infinite extent (below). The horizontal line $\Omega_\Lambda = 0$ roughly distinguishes between an ‘unbound’ universe that will expand forever (above) and a ‘bound’ universe that will eventually recollapse (below). The special point $(\Omega_m, \Omega_\Lambda) = (1, 0)$ corresponds to the simplest model, termed Einstein–deSitter; unless the universe exactly fits this model, it evolves away from this point as it expands. If $\Omega_\Lambda > 0$, the universe eventually crosses the line $\Omega_\Lambda = 0.5\Omega_m$, which separates a universe whose expansion decelerates by attraction (below) or accelerates by repulsion (above).

New techniques to measure distances of supernovae (type Ia) at large distances (cosmological redshifts 0.5–1), where the large-scale curvature of space–time plays a noticeable role, enable a classical cosmological test based on how the time dependence of the Hubble relation between velocity and distance depends on the cosmological parameters⁸. The corresponding, inclined (blue) confidence limits in Fig. 1 are based on the results of the Supernova Cosmology Project¹, which are fully consistent with the findings of the High-*z* Supernova Search Team². The allowed region in the parameter plane is an elongated stripe, crudely approximated by $0.8\Omega_m - 0.6\Omega_\Lambda = -0.2 \pm 0.1$ (1σ errors). Based on this result alone, a nearly flat universe is likely, with a broad maximum to the probability near $(\Omega_m, \Omega_\Lambda) = (0.3, 0.7)$, but an open model, of $(0.1, 0)$, for example, is still allowed at the 2% confidence level. An orthogonal constraint is required in order to remove the degeneracy between Ω_Λ and Ω_m .

Here we show that such a constraint is provided by current data of

‘peculiar’ velocities of galaxies on scales of ~ 300 million light years. These velocities, which are produced by gravity due to local fluctuations about the mean mass density, depend also on the value of the mean density itself, Ω_m . The constraints on Ω_m from these velocities are almost independent of Ω_Λ , but combined with the inclined supernova constraints, any bound on Ω_m effectively serves as a bound on Ω_Λ .

We use two recent catalogues of galaxy peculiar velocities. One, named Mark III (ref. 9), contains distances for $\sim 3,000$ galaxies within $\sim 70 h^{-1}$ Mpc; the other, named SFI (refs 10, 11), consists of $\sim 1,300$ spiral galaxies with a more uniform sampling in a similar volume. In order to obtain the peculiar velocity of a galaxy, its total velocity is measured using the Doppler redshift, and its distance is separately inferred using the so-called Tully–Fisher method, with an error of 15–21%. The desired peculiar velocity along the line of sight is obtained by subtracting, from the total velocity, the Hubble expansion velocity at the galaxy distance. The peculiar velocities are carefully corrected for systematic errors^{7,12}.

The POTENT method^{12,13} assumes that the peculiar velocities were generated by gravity and that they therefore represent a potential flow on large scales. This allows a recovery of the three-dimensional velocity field of galaxies in our local cosmological neighbourhood. For an assumed value of Ω_m , the underlying field of mass-density fluctuations can be extracted, using slightly non-linear approximations to the relation between velocity and density fluctuations within the theory of gravitational instability. Assuming also that the initial fluctuations were a gaussian random field, direct dynamical constraints on Ω_m can be obtained without appealing to the observed spatial distribution of galaxies. These constraints are thus independent of the unknown relation between galaxies and the underlying mass density (termed ‘biasing’). Several different methods applied to the POTENT Mark III fields consistently yield a lower bound of $\Omega_m > 0.3$ at the 99% confidence level, marked by a vertical red line in Fig. 1. One method constrains Ω_m based on the large diverging outflow recovered in the vicinity of a large nearby underdense region (void), using the fact that even an empty void cannot induce outflows as large as those observed if the density outside it is too low³. A second method uses the deviations of the initial fluctuations from a gaussian distribution, when derived from the velocity data, assuming a value of Ω_m that is too low⁴. A third method derives Ω_m based on the gravitationally-induced deviations from gaussian distribution of the present-day velocity-divergence field⁵. Similar constraints confirming the lower bound of $\Omega_m > 0.3$ are currently being obtained from the SFI data.

In a new analysis^{6,7}, not based on POTENT, we have applied a maximum-likelihood analysis to the Mark III and SFI peculiar velocities to determine cosmological parameters. It is done via a parametric model for the mass-density fluctuation power spectrum P_k , which measures the distribution of power among different length scales, proportional to k^{-1} . This analysis uses linear gravitational theory and assumes that both the fluctuations and the errors are gaussian. The model used is of the popular cold dark matter (CDM) model family; P_k is normalized¹⁴ by the fluctuations in the cosmic microwave background (CMB) as measured on very large scales by the COBE satellite. The result provides constraints on permissible combinations of three parameters: Ω_m , the power index n (where $P_k \propto k^n$ on large scales), and the Hubble expansion constant h . These parameters enter via the shape of P_k as well as from the geometry and dynamics of space–time. The constraints obtained from the two data sets turn out to be similar; they define a two-dimensional surface in the Ω_m – h – n space. In the case of a flat cosmology this surface can be crudely approximated by $\Omega_m h_{65}^3 n^2 \approx 0.58 \pm 0.12$ (where h_{65} is the Hubble constant in units of $65 \text{ km s}^{-1} \text{ Mpc}^{-1}$, and the errors refer to 90% uncertainty).

The obtained P_k is determined by the velocity data (on scales of $\sim 50 h^{-1}$ Mpc) and is not much affected by the COBE normalization (at $\sim 1,000 h^{-1}$ Mpc); a similar P_k is reproduced to within a few per

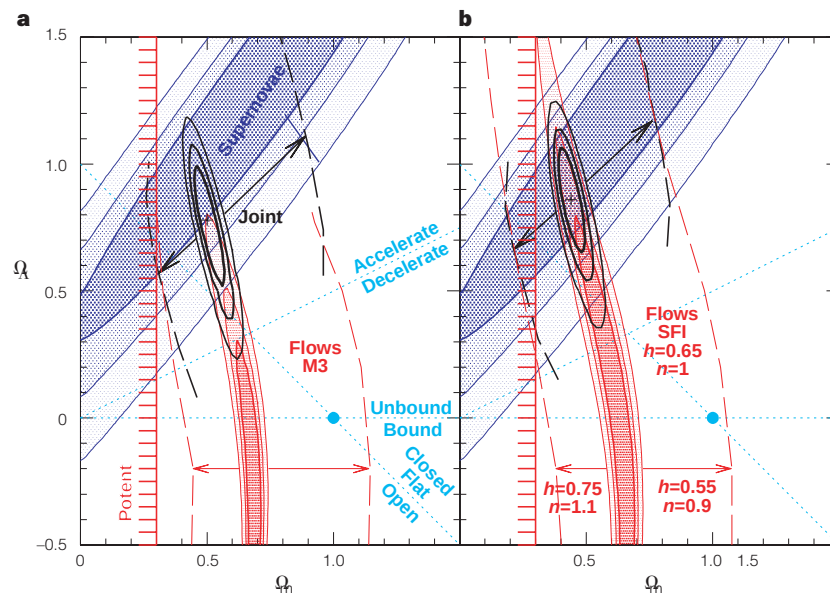


Figure 1 Constraints in the Ω_m – Ω_Λ plane, showing relative confidence limits of 68, 90 and 99%. The inclined contours (blue in **a** and **b**, that roughly constrain $0.8\Omega_m - 0.6\Omega_\Lambda$, arise from the global geometry of space–time based on supernovae as distance indicators¹. The lower bound $\Omega_m > 0.3$ (red) represents a 99% confidence limit based on several different studies of peculiar velocities using POTENT reconstruction, independent of the biasing relation between galaxies and mass^{3–5}. The almost vertical contours (red) arise from the peculiar velocities of the **a**, Mark III, and **b**, SFI catalogues, based on a likelihood analysis which assumes a parametric mass–power spectrum of the cold dark matter model family with COBE normalization on large scales^{6,7}. The central contours shown are for a standard case of fixed $h = 0.65$ and $n = 1$; the uncertainties in these values are represented by the outer 99% contours on both sides (long dashed red),

computed for the extreme cases of fixed $(h, n) = (0.55, 0.9)$ and $(0.75, 1.1)$. These encompass a conservative range of non-negligible likelihood based on the flow data. The strong bounds from flows are on Ω_m ; the apparent upper bound on Ω_Λ arises indirectly from the COBE normalization and is very uncertain; the contour can extend further in the Ω_Λ direction, allowing significant overlap with the supernova confidence region. The corresponding joint (relative) confidence limits from supernovae and flows are shown (black contours); they are almost the same for Mark III (**a**) and SFI (**b**). The combined constraints thus favour an unbound and roughly flat universe with comparable contributions from the cosmological constant and mass density. A universe with very low Ω_m and zero cosmological constant is ruled out.

cent when the amplitude on large scales is left as a free parameter. Also, the result is not dominated by the assumed distance errors; P_k is reproduced to better than 10% when an error model with free parameters is incorporated in the likelihood analysis⁷. This robustness is within the 1σ error limits of the likelihood analysis.

The peculiar velocities have also been analysed by methods that incorporate the spatial distribution of galaxies. For example, a comparison of the Mark III velocities and the IRAS 1.2 Jy redshift survey¹⁵ yields results which are roughly consistent with the CDM model of $\Omega_m \approx 0.5$ favoured by the P_k analysis of flows reported above. Our current bounds on Ω_m from flows also partly overlap with constraints from the evolution of galaxy clusters¹⁶ ($\Omega_m = 0.45 \pm 0.2$). However, a comprehensive joint analysis of all the available constraints is beyond the scope of this work. In particular, a thorough interpretation of the results involving redshift surveys (which span a noticeable range) must include a full discussion of the non-trivial biasing relation between galaxies and mass¹⁷, and of the complex systematic effects involved in these different studies. Instead, we choose to focus here on one set of constraints, the dynamical constraints from flows, combined with the supernova constraints.

In order to obtain the desired constraints in the Ω_m – Ω_Λ plane, we have applied the P_k likelihood analysis to the Mark III and SFI velocities, using the COBE-normalized¹⁸ CDM models with varying Ω_m and Ω_Λ that now span the whole parameter plane. The velocities are expected to be insensitive to the value of Ω_Λ (ref. 19), but a weak Ω_Λ dependence occurs as a result of the COBE normalization imposed. This is responsible for the slight left bending of the red vertical likelihood ridges (Fig. 1), and the apparent upper bounds on Ω_Λ . The 1σ error in the COBE normalization translates to additional uncertainties of $\pm 6\%$ in Ω_m and $\pm 20\%$ in Ω_Λ , which are

of the order of the uncertainties displayed by the likelihood contours shown. Thus, the COBE errors would tend to stretch the likelihood contours vertically along the Ω_Λ axis, weakening the apparent upper bounds on Ω_Λ .

As the velocity data favour an extended two-dimensional surface in the Ω_m – h – n space, the likelihood analysis cannot determine the three parameters simultaneously; two of them should be fixed initially. We adopt as our standard case the simplest, scale-invariant ($n = 1$) initial spectrum, and $h = 0.65$ (as favoured by nearby supernova data²⁰). We crudely estimate a $\pm 15\%$ uncertainty in the Hubble constant²¹, and $\pm 10\%$ in the power index²². In order to illustrate the sensitivity of the constraints to these parameters, we have also determined Ω_m and Ω_Λ for two extreme cases of h and n values. The outer 99% contours are shifted accordingly in Fig. 1.

The likelihood ridge of SFI extends into higher values of Ω_Λ than for Mark III. The independent constraints from flows (using P_k) and supernovae overlap significantly for SFI, but only near the 90% contours for Mark III with $(h, n) = (0.65, 1)$ (which improves when COBE errors are considered or if $n > 1$ or $h > 0.65$). The large uncertainty in the upper bound on Ω_Λ indicates that the degree of overlap should not be interpreted as indicating either consistency or inconsistency between the velocity and supernova data and the assumed standard cosmological model.

A joint parameter estimation from supernovae and flows is therefore meaningful, although it is limited to relative likelihoods; a measure of absolute probabilities is not straightforward, despite the seemingly acceptable goodness of it. The black joint contours (Fig. 1) are computed by multiplying the two likelihood values at each point, having assumed that the data are independent. The most likely joint values (shown as crosses in Fig. 1) for the supernovae and the combined Mark III and SFI dataset are $(\Omega_m, \Omega_\Lambda) = (0.5, 0.8)$,

while the conservative joint 99% confidence limits allow Ω_m values in the range 0.3–0.9 and Ω_Λ values in the range 0.1–1.4. The constraints obtained separately from Mark III and SFI are very similar.

The constraints from local flows thus remove the degeneracy in the constraints from the global-geometry test based on supernovae (and vice versa), and help rule out an open model with zero cosmological constant. A nearly flat universe, with comparable contributions of matter and cosmological constant to the total energy density, is likely. The favoured model is therefore an unbound universe that will accelerate forever, although it is still impossible yet to determine whether the global geometry is flat, open or closed.

Such comparable contributions from the mass density and the cosmological constant represent a puzzling fine tuning, for example because the two parameters, Ω_m and Ω_Λ , are expected to vary with time in opposite senses. The standard theory expects the cosmological constant either to vanish or be larger by many orders of magnitude^{8,23}. Perhaps entropic arguments may be needed to explain such a fine tuning. Although the observed constraints are not yet finally confirmed, they already seem interesting enough to pose a serious challenge to theoretical physics.

Other constraints in the Ω_m – Ω_Λ plane are worth mentioning in this framework. Constraints consistent with the supernova ridge but of larger uncertainty arise from the age of old star clusters²⁴ versus the Hubble expansion rate. Constraints of orthogonal orientation, roughly on $\Omega_\Lambda + \Omega_m$, can be deduced from the acoustic peaks in the sub-degree angular power spectrum of fluctuations in the CMB, as observed from balloons and from the ground^{22,25–29}. Two CMB satellites planned for the next decade, MAP and Planck, are expected to provide more accurate constraints^{27,30}.

Future peculiar-velocity data are expected to improve the accuracy of the constraints. In addition to many new velocities based on distance indicators of the Tully–Fisher type, the most promising sources of large-scale peculiar velocities in the future will probably be the siblings of the same supernovae discussed above, but at low redshifts; their distances can be measured with 5–10% accuracy out to large distances and their sampling density is limited only by the patience of the observers. □

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A metal complex that binds α -amino acids with high and predictable stereospecificity

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Molecular recognition is the key step in a wide range of controlled separation and chemical transformation processes, with enzymes performing this task with an unsurpassed degree of selectivity. Enzymes contain only 20 simple amino acids, yet it remains difficult to rationalize or even predict these stereospecific recognition events. Nonetheless, the rational design of receptors able to recognize amino acids stereospecifically is attracting considerable interest because therapeutic drugs, that may be developed from chiral amino acid intermediates, are increasingly required in enantiomerically pure form¹. Early work^{2–4} has stimulated the development of efficient receptors based on small molecules^{5–8}, but binding of amino acids with high and predictable stereospecificity remains difficult to achieve. Directed molecular evolution⁹, on the other hand, does select for RNA sequences or antibodies that bind amino acids with high specificity^{10–12}, but typically without providing insights into the molecular recognition mechanisms involved. Here we show that a rationally

designed metal complex formed from a trivalent cobalt ion and a tetradentate ligand binds natural amino acids, including the simple yet challenging amino acid alanine, with high and predictable regio- and stereospecificity. We expect that our approach will allow the binding as well as separation and stereospecific catalytic formation of its target amino acids.

There is much interest in rationally designing chiral metal complexes that bind or transform their targets, whatever they may be, with high and predictable stereospecificity. However, it has been a challenge to rationalize even the sense of the observed stereospecificity in simple binding or catalytic processes^{13–15}.

Octahedral metal complexes have often been used for stereospecific recognition of natural amino acids^{16–19}. In order to control the regiospecific and stereospecific recognition of amino acids in a predictable way, we were interested in designing a chiral tetradentate ligand that forms only one isomer of an octahedral metal complex. Compound **1**, an unnatural α -amino acid, can form only one configurational isomer of an octahedral metal complex (**2**) due to the relative position of the four coordinating atoms (Fig. 1). Furthermore, molecular mechanics calculations show that **2** essentially exists as one conformational isomer. The [1,2,3] bicyclic portion of **2** (sharing the amino group bridge) is conformationally rigid, and the other five-membered ring in the complex (containing the dimethyl amine group) has only two possible conformations of which the one shown (**2**) is computed to be about a million times more populated than the other at equilibrium at 25 °C. Indeed, the crystal structure of the dinitro Co(III) complex formed from **1** is that of **2** (Fig. 2a).

In designing the tetradentate ligand (**1**) we reasoned that the carboxylate group of the ligand might prefer to coordinate *trans* to the carboxylate group of alanine (Fig. 1, **3a**) as has been shown for square planar bis-glycine complexes of Ni(II), Cu(II) and Zn(II) (ref. 20). Once regiospecific coordination of alanine is controlled, stereospecific coordination of alanine may be considered. Alanine forms a five-membered ring upon chelation to the metal complex (**3a**). In such five-membered rings the alanine methyl group is expected to occupy the equatorial position to avoid steric congestion with the coordinated atoms²¹. The α -H of the chelated alanine therefore has to occupy one of the two axial positions (Fig. 1, **3a** or **3b**). If one of these two axial positions is blocked with the dimethyl amine group of the tetradentate ligand, stereospecific recognition of alanine should result (that is, **3a** should be favoured over **3b**). Before synthesizing the tetradentate ligand, we carried out molecular mechanics calculations on **3a** and **3b**. In accordance with the

above reasoning, the computed energy value for **3a** is lower than that for **3b** by about 3 kcal mol⁻¹. It is also evident from computation that the α -H of alanine in **3b** comes in direct contact with one of the two methyl groups in the dimethyl amine group.

Compounds **1** and **2** were synthesized as racemic mixtures according to the path in Fig. 3. Addition of an equivalent amount of ($\pm$)-alanine to a racemic mixture of the dichloro cobalt complex (prepared from **2**) gave two main products **3a** and **3b** (each as a

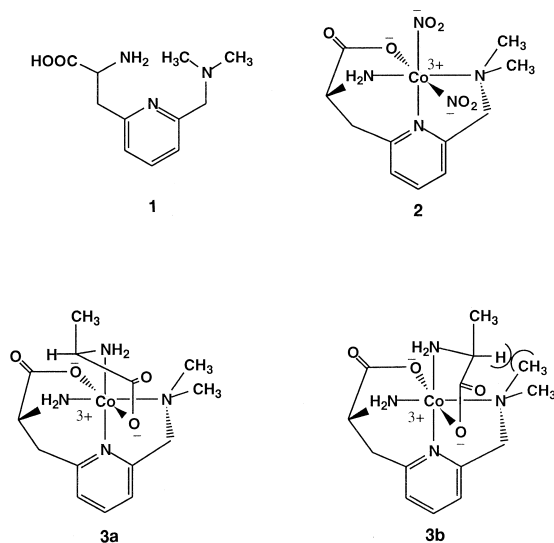


Figure 1 Structures of free chiral ligand (**1**), chiral cobalt complex (**2**) and alanine bound chiral cobalt complexes (**3a** and **3b**).

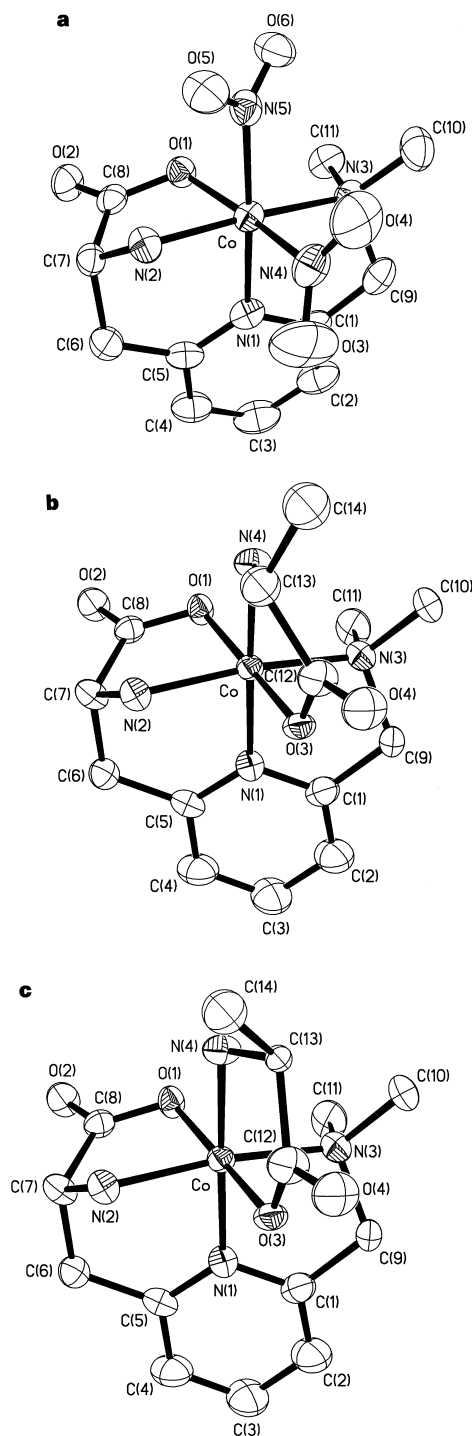


Figure 2 X-ray structures of cobalt complexes **2** (in panel a), **3a** (in panel b) and **3b** (in panel c): ORTEP representation with ellipsoids at the 20% probability level. Crystal structural data for purple square plates of a mixture of **3a**(ClO₄) and **3b**(ClO₄): orthorhombic, space group P2₁2₁2₁, $a = 9.620(3)$ Å, $b = 10.732(3)$ Å, $c = 19.710(4)$ Å, $Z = 4$; $R = 0.0461$, $GOF = 0.787$.

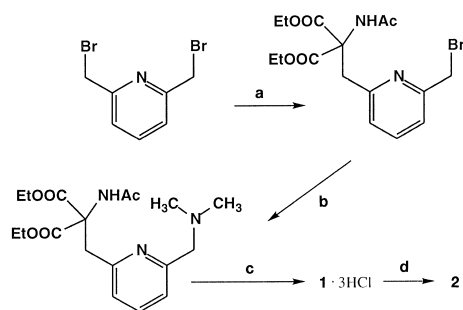


Figure 3 Reagents and conditions for the synthesis of **1** and **2**. **a**, Diethyl acetaminomalonate (1 eq.), NaH, TFH, 60 °C; **b**, Me₂NH in CH₃CN; **c**, 6 N HCl, reflux for 12 h; **d**, (i) 4 eq. NaOH (ii) [Co(III)(NO₂)₆]Na₃, 20 °C for 2 h.

racemic mixture). This mixture of **3a** and **3b** (Fig. 2b, c) was crystallized and subjected to crystallographic analysis revealing one racemic compound with temperature independent disorder for positions C13 and C14. We interpret this disorder to be caused by the presence of both diastereomers in the unit cell (data analysis gave ~70% **3a** and ~30% **3b** in the crystal mixture). The X-ray structures of **3a** and **3b** (Fig. 2b, c) are precisely in accord with the rational design. The two carboxylate oxygens (O1 and O3) in **3a** and **3b** are positioned *trans* to each other establishing regiospecific binding of alanine. The alanine methyl groups (C14) in **3a** and **3b** are in equatorial positions as predicted. The C13 hydrogen (α -H of alanine) of **3b** points towards the C10 carbon while that of **3a** points away from the C10 carbon. All of these features are in accord with the rational design.

Results of 500 MHz ¹H NMR scanning of the above crystalline mixture of **3a** and **3b** (again both racemic) indicate that the ratio of the two diastereomers in the crystal is about 60/40 (Fig. 4a); in reasonable agreement with the ratio obtained from the crystallographic analysis (70/30). Most of the signals for **3a** overlap with those for **3b** but two of the three methyl peaks (*a* and *c* peaks) are well enough separated to allow observation of the 60/40 diastereomeric ratio (see Fig. 4a). The *a* peak corresponds to one of the methyl groups of the tetradentate ligand and the *c* peak corresponds to the alanine methyl group.

Interestingly, **3b** epimerizes to **3a** in basic D₂O (Figs 4, 5). The *c*(**3a**) doublet rapidly disappears (Fig. 4a, b) to completion with a concomitant increase in the *c*(**3a-D**) singlet. This indicates that essentially all of **3a** has been deuterated. The *c*(**3b**) doublet also decreases but much more slowly than the *c*(**3a**) doublet. Conversion of **3b** to **I** (Fig. 5) is expected to be slower than conversion of **3a** to **I** (Fig. 5) since the α -H of alanine in **3b** is sterically more hindered. Similarly, deuteration of **I** is expected to take place more rapidly from the less hindered side to give more **3a-D** than **3b-D**. Consistent with this interpretation, we do not observe a signal for the *c*(**3b-D**) singlet (Fig. 4b). Although the signal due to a small amount of **3b-D** may be buried, integration of all the peaks in Fig. 4b (the integration ratio is 24:52:11:65 for peaks downfield to upfield with the two most upfield peaks integrated together) reveals that the amount of **3b-D** is unlikely to be more than 5% that of **3a-D**. It is clear from the above that the metal complex formed from the *L*-stereoisomer of **1** binds to the *L*-stereoisomer of alanine under thermodynamic control. The deuteration reaction shown in Fig. 5 implies that alkylation of **3a** and **3b** may also take place stereospecifically.

The fundamental concepts used here to develop the chiral metal complex for regiospecific and stereospecific recognition of alanine should be applicable for other bidentate α -H-amino acids. In order to investigate this possibility, we synthesized the receptor metal complex in enantiomerically pure form with a methyl group at the position of the α -hydrogen. This receptor in the *R*-form (which cannot be racemized due to the methyl group) was reacted with

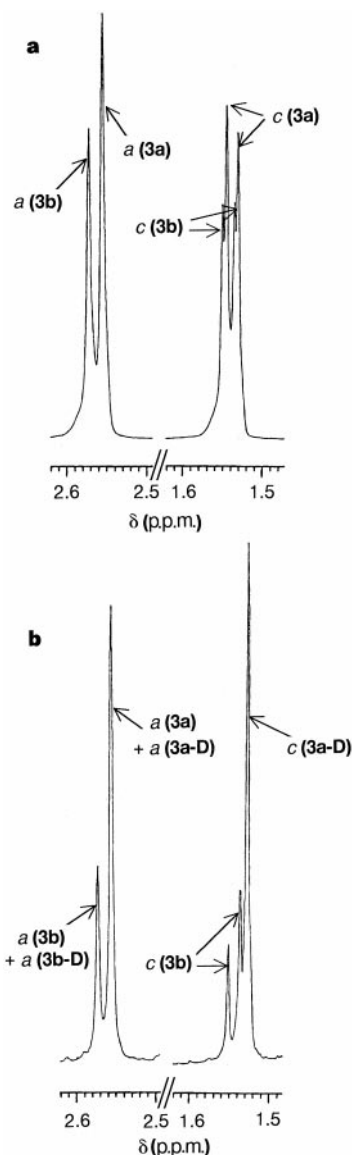


Figure 4 Characterization of the epimerization of **3a** and **3b**. Shown are 500 MHz ¹H NMR signals of one of the methyl groups of the tetradentate ligand (peak *a*) and the methyl group of alanine (peak *c*) in **3a** and **3b** (3 mM) before (panel **a**) and after (panel **b**) partial epimerization in 5 ml of 99.9% D₂O (5 mM NaOD, 50 h at 25 °C). In **a**, the integration ratios of *a*(**3a**)/*a*(**3b**) and *c*(**3a**)/*c*(**3b**) are both about 60/40. After partial epimerization (**b**), all of the *c*(**3a**) doublet has been converted to the *c*(**3a-D**) singlet. Also, peaks *a*(**3b**) and *c*(**3b**) have decreased but there is no evidence for *c*(**3b-D**).

glycine, *L*-alanine, *D*-alanine, *L*-phenylalanine, *D*-phenylalanine, *L*-tryptophan, and *D*-tryptophan. Essentially one complex formed in each of the seven reactions. The seven products were purified by ion exchange chromatography and treated with base in D₂O (see Supplementary Information). The α -hydrogens of the three *D*-amino acid products exchanged rapidly and completely with little or no observable epimerization. In contrast, the α -hydrogens of the three *L*-amino acid products exchanged slowly with concomitant epimerization. We were not able to fully deuterate the *L*-amino acid complexes due to competing decomposition reactions. Thus, the *R*-form of the receptor not only binds more tightly to the *D*-forms of the amino acids but also converts the *L*-forms to the *D*-forms. Note that amino acids with side chains that can interact strongly with the receptor (by coordination or H-bonding) may behave differently from the above mentioned amino acids. Nevertheless, this study clearly sets the framework for stereospecific recognition of amino

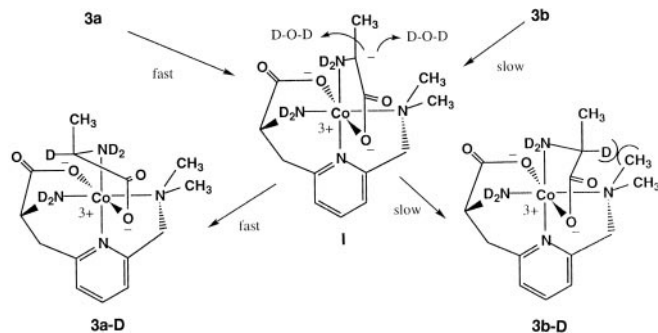


Figure 5 Mechanisms for deuteration/epimerization of **3a** and **3b** to **3a-D** and **3b-D**.

acids and, by implication, of structurally related molecules, such as chiral glycols, 1,2-diamines, 1,2-aminoalcohols, and α -hydroxy acids.

The X-ray crystallographic (Fig. 2) and ^1H NMR (Fig. 4) data show that coordination of alanine to our metal complex takes place with unprecedented regiospecificity and stereospecificity in accord with the design rationale. The regiospecificity is apparently controlled by electrostatic effects while the stereospecificity is controlled by steric effects in a highly predictive manner. This approach thus provides detailed structural insights into general separation of bidentate α -H-amino acids into D and L forms with a single chiral metal complex. Such a complex may also be useful as a catalyst for synthesizing α -H amino acids²² or hydrolysing^{23,24} their esters with predictable stereochemistry. □

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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Cloud albedo enhancement by surface-active organic solutes in growing droplets

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Understanding the properties of clouds in the Earth's atmosphere is currently limited by difficulties at the fundamental level of adequately describing the processes of cloud droplet nucleation and growth. Small changes in droplet population may significantly influence cloud albedo¹ as well as formation of precipitation. Models of cloud formation based on laboratory studies with idealized composition of nuclei suggest that organic solutes significantly lower surface tension²—one of the parameters determining droplet population—but the lack of data on composition and properties of the organic material in the atmosphere precludes realistic laboratory or model studies. Here, we report measurements on vacuum-evaporated samples of cloud water from the Po Valley, Italy, that show a large decrease in surface tension, by up to about one-third relative to pure water, for realistic concentrations of organic solutes expected to exist in growing droplets. Such large surface-tension changes, if they occur in cloud droplets near the critical size for nucleation, lead to an increase in droplet population and hence in cloud albedo. The error produced in ignoring this effect is estimated to be comparable to other calculated direct and indirect influences of aerosols on scattering and absorption of solar radiation³.

Traditional equilibrium (Köhler) theory⁴ predicts that small (sub-micrometre) aerosol particles which are partially or completely water soluble act as cloud condensation nuclei (CCN) by lowering the vapour pressure and thus overcoming the requirement for large supersaturations caused by the small radius of curvature of the incipient droplets. The controlling variables would then be particle size and water-soluble mass; however, when measured, these alone do not accurately predict the CCN population¹. Recently, additional variables have been proposed as factors that may also influence cloud droplet formation and properties. These variables include CCN solubility², droplet growth kinetics⁵ and water-soluble gases⁶. Surface tension (σ), which is one of the factors that controls the vapour pressure of small droplets, is the consequence of intermolecular attractive forces tending to minimize the surface area of the liquid. Water has a high surface tension because of strong hydrogen bonds, the effect of which is diminished by the presence of surface-active solutes (surfactants).

Surface tension enters into the description of cloud droplet

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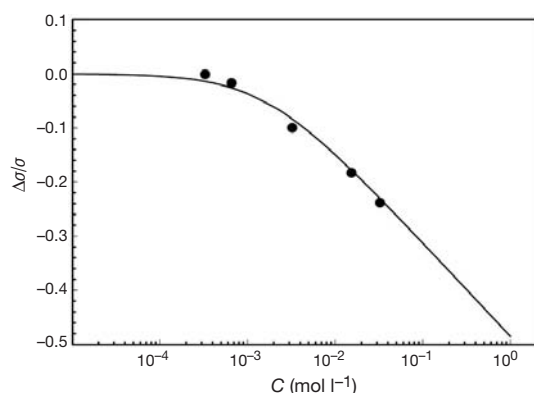


Figure 1 Measured change of surface tension. Samples of Po Valley fog water with respect to pure water, plotted versus dissolved organic carbon concentration (C). The curve is the function $\sigma = 72.8 - 0.01877 \ln(1 + 628.14C)$, derived by fitting the equation of Szyszkowski–Langmuir¹⁵ to the data.

nucleation in the Kelvin term of a simplified form of the Köhler equation⁴ for supersaturation (S) over a solution droplet of radius r : $S \approx a/r - b/r^3$, where a is proportional to σ and b includes the solute amount in the negative Raoult term. Here $a = 2M\sigma/RT\rho$ and $b = 3\nu nM/4\pi\rho$ where M is the molecular mass of water, σ is the surface tension at an air/water interface, R is the gas constant, T is the temperature, ρ is the water density, ν is the van't Hoff factor (approximately the number of moles of ions produced per mole of solute) and n is the number of moles of solute. When $dS/dr = 0$, the droplet is said to be at a critical size for growth (r_c) with droplets larger than r_c growing and smaller droplets staying at a size that is in equilibrium with the ambient S . This critical size corresponds to a critical supersaturation: $S_c \propto a^{3/2}b^{-1/2}$, such that S_c depends on $\sigma^{3/2}$. It is at droplet sizes and solute concentrations below and close to the critical size ($r_c \approx 0.1 \mu\text{m}$ to a few micrometres) that surface tension would most effectively influence whether or not a particle activates and grows into a cloud droplet (typically 5–10 μm).

Prior measurements of the surface tension of rain water, melted snow and dispersions in water of sampled atmospheric particles concluded that the contribution of either weakly soluble or soluble materials were too small to have a significant influence on cloud microphysics⁷. However, these studies involved concentrations corresponding to fully grown cloud droplets ($\sim 10^{-7}$ – 10^{-5} M), whereas the concentrations of total solutes within incipient droplets near r_c are estimated to be much higher (10^{-4} – 10^{-2} M or more)⁸. Many organic compounds found in the atmospheric aerosol are commonly listed as insoluble⁹ but in fact have solubilities in the 10^{-5} – 10^{-2} M range and would therefore be available as solutes near r_c .

A simple method of recreating the conditions that exist for droplet sizes near r_c is to vacuum evaporate collected fog or cloud samples by factors of 10, 100, or more while measuring σ . This provides a range of concentrations and also demonstrates to what degree real atmospheric solutes can cause surface tension to change and whether the concentration of surfactants is sufficiently high. Figure 1 shows such data from fog water collected in the Po Valley of Italy, revealing a relative decrease of surface tension ($\Delta\sigma/\sigma$) of 30% at the highest concentrations. Collected volumes of ~ 1 litre were evaporated in steps, with aliquots removed sequentially for measurement of surface tension using an oscillating bubble tensiometer¹⁰. Chemical analyses of these samples showed that the solutes were typically 80% inorganic salts and 20% organic matter. The latter is a complex mixture of oxygenated compounds, mostly acidic (70–80%), but also containing neutral polyols and polyglycolethers. The acidic mixture is largely composed of aliphatic hydroxylated compounds of low molecular mass, but polyacidic compounds of high

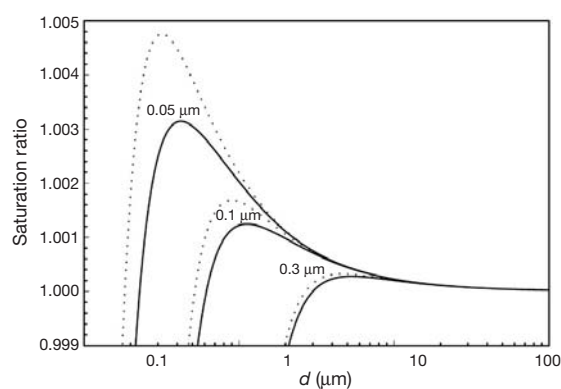


Figure 2 Köhler curves given by a modified Köhler equation². The calculations were done for the same chemical composition without surfactants (dotted line) and with surfactants (solid line). The dry aerosol particle sizes were 0.05, 0.1 and 0.3 μm and the chemical composition was considered to be 70% soluble (50% inorganic salts and 20% organic matter). Here d is droplet diameter, and the saturation ratio equals $S + 1$.

molecular mass represent an average 25% of the organic solute. The above classes of organic compounds, the polyacids in particular, are known surfactants. Although the Po Valley is known for its polluted winter fogs, the organic fraction and concentrations in the aerosol are similar to air in or downwind of other large agricultural/industrial regions^{11,12}. The similarity of these field data to those obtained using model compounds, especially for mixtures of inorganic salts and organic oxygenates, is striking². Thus, the possibility exists that similar results would obtain over a significant fraction of the Earth, at least in the vicinity of continents.

One way to assess the possible influence of the decrease of surface tension is to generate Köhler curves ($S \approx a/r - b/r^3$) considering the same chemical composition with and without surfactants. Figure 2 illustrates the consequences of the high (10^{-4} – 10^{-2} M) concentration of surface-active organic solutes below and in the vicinity of r_c . Dry particle sizes were chosen to match roughly the range of CCN dry radii found in the atmosphere (0.05–0.3 μm). As the aerosol population is strongly skewed toward smaller sizes¹², the decrease of surface tension for small particles probably dominates the overall system.

Given the possibility that this kind of organic aerosol composition and concentration can exist over large regions, we can make a simple assessment of the resultant climate forcing from a widespread change of σ . No exact relationship exists between cloud droplet population (N) and supersaturation (S), but the dependence relation $N \propto S^k$, with $k \approx 0.5$, is often used¹³. Hence, $\Delta N/N \approx k\Delta S/S$ for small changes. Next we recognise that the measured decrease of σ has the same effect on cloud microphysics as would leaving σ at the value for pure water and increasing the atmospheric supersaturation S . The above equation for S_c then yields (for the effective increase in S): $\Delta S_c/S_c \approx -(3/2)\Delta\sigma/\sigma$. Hence $\Delta N/N \approx -(3/4)\Delta\sigma/\sigma$, which allows a direct assessment of possible climate forcing caused by changes in surface tension. A 30% decrease in σ results in an $\sim 20\%$ increase in N . To the extent that the amount of liquid water in clouds is controlled primarily by the amount of atmospheric cooling, this increase in N results in a decrease of the mean size of droplets of $\sim 6\%$.

A simple diagnostic estimate illustrates the potential for changing droplet populations and hence cloud albedo. The above 20% increase in N yields a change in top-of-atmosphere albedo locally of $\sim 1\%$. If that were to occur within all stratus clouds, a global mean forcing of almost -1 W m^{-2} would arise. This is an upper bound, because not all stratus clouds could be affected¹⁴. Thus, surface tension changes from pure water are estimated to have potential effects of the same magnitude as those of droplet growth kinetics⁵, CCN solubility² and water soluble gases⁶.

Cloud droplet coalescence, development of precipitation and (ultimately) cloud lifetime are also influenced by droplet populations and thus by surface tension changes. But, in order to quantify them, a more quantitative description of the process of cloud droplet nucleation is needed. It is also necessary to put this result in the context of all the other natural and anthropogenic factors that may change cloud albedo and cause climate forcing; these factors do not operate independently of each other or independently of supersaturation. These interconnections pose challenges for both the measurement and the modelling communities. Finally, given the sensitivity of cloud albedo to surface tension, it would be useful to understand changes in the latter that are a result of human activity. □

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Control of the location of the volcanic front in island arcs by aqueous fluid connectivity in the mantle wedge

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The water released from descending oceanic lithosphere is thought to have an important role in subduction-zone magmatism, as this water might trigger partial melting of the mantle wedge above the subducting plate^{1–8}. If, however, there is incomplete wetting of mineral grain boundaries in the mantle (that is, the dihedral angle⁹ at the triple junctions between grains

is more than 60°), then the water would not form an interconnected network and might instead be trapped as interstitial fluid in the mantle peridotite. The water would then be transported to deeper parts of the mantle rather than triggering partial melting. Here we use dihedral-angle data to estimate the connectivity of an aqueous fluid phase in a model upper-mantle mineral assemblage (forsterite) at pressures from 3 to 5 GPa (corresponding to depths of ~80–150 km). By combining these data with previous results^{10,11}, we find that the dihedral angle is greater than 60° at low pressure and temperature (<1,000 °C at 2 GPa and <800 °C at 4 GPa) and lower than 60° at higher pressures and temperatures, suggesting that wetting is incomplete below these conditions. This indicates that the connectivity of water in hydrous upper-mantle peridotite at convergent plate boundaries might control the position of the volcanic front in island arcs.

High-pressure experiments were performed with the multi-anvil apparatus (ERI-2000) at the Earthquake Research Institute, University of Tokyo. The starting materials, Mg₂SiO₄ + 5.0 wt% H₂O, were prepared from a mixture of reagent-grade Mg₂SiO₄, SiO₂ and Mg(OH)₂ in Mg₂SiO₄ + H₂O stoichiometry. The starting material was placed in platinum capsules that were welded at both ends. The aqueous fluid supplied by Mg(OH)₂ is predominantly H₂O, rather than H₂, at the experimental conditions because the oxygen fugacity of the furnace assembly employed in this study is more oxidizing than the wüstite–magnetite buffer¹¹. Details of the experimental procedure are described elsewhere¹¹.

The run product consisted of semi-sintered aggregates of forsterite (Fig. 1). To characterize the wetting behaviour, dihedral angles⁹ (θ) at aqueous fluid–forsterite–forsterite triple junctions were measured with a scanning electron microscope (SEM). The measurements were conducted as described¹². We adopted the median angles¹³ of populations of about 180 observations as the true dihedral angles.

Our experimental results are shown in Fig. 2. We obtain median angles of 62° (Fw-17) and 57° (Fw-18) from the distribution of apparent dihedral angles at 3 GPa and 5 GPa, respectively. The solid lines in the figure show the theoretical distribution¹³ for monomineralic isotropic systems with dihedral angles of 62° and 57°, respectively. The observed distributions are wider than the theoretical ones, perhaps because forsterite is anisotropic.

In a strict sense, the isotropic equilibrium model does not describe adequately the distribution of a fluid phase in mantle minerals^{14,15}. This is confirmed by the observation that some of the olivine grains have flat crystalline interfaces which are controlled by crystal structure rather than by minimization of surface area. From theoretical analysis of anisotropic systems¹³, however, the true

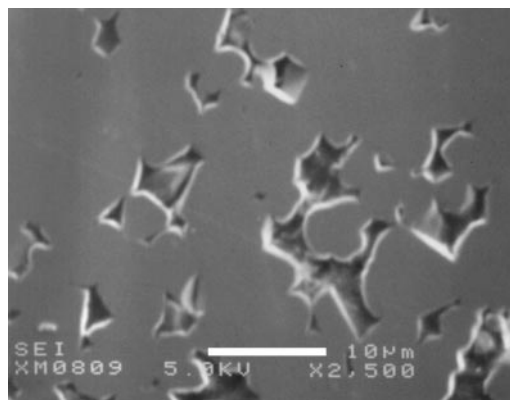


Figure 1 Secondary electron photomicrograph of the polished sample at 3 GPa and 800 °C. Fluid-filled grain boundaries are filled with epoxy (nearly black). The fine-grained materials set in epoxy are considered to be quenched phases from aqueous fluid. The morphology of grain-edge fluid is preserved upon quench because the grains are relatively well sintered. Scale bar, 10 μm.

angles should be within 10° of the median value. We therefore used the median dihedral angles to estimate the wetting behaviour of the system.

The dihedral angle θ decreases with increasing pressure and temperature (Fig. 3), as also observed at lower pressures of 0.5–2.0 GPa (ref. 10). The $(\partial\theta/\partial T)$ does not vary with pressure between 1 and 5 GPa. The critical θ of 60° (see discussion in legend to Fig. 2) is attained near 2 GPa at $1,000^\circ\text{C}$ and near 4 GPa at 800°C (Fig. 3b).

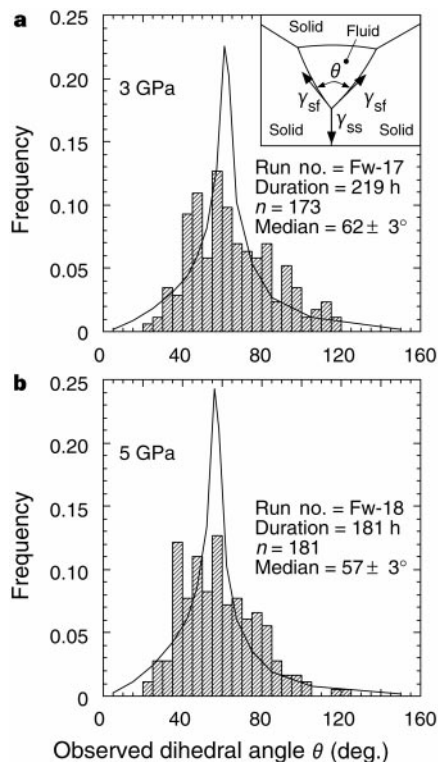


Figure 2 Apparent distribution of dihedral angles at aqueous fluid–forsterite–forsterite triple junctions at 800°C . **a**, 3 GPa; **b**, 5 GPa. The theoretical distributions¹³ for a monomineralic, isotropic system with dihedral angle of 62° (**a**) and 57° (**b**) are also shown for comparison. The quoted error is the 95% confidence interval around the median, calculated as described^{28,29}. n indicates the population of the observed angles measured. Because the dihedral angle does not change significantly after 50 h of experimental duration¹¹, the durations are considered sufficient to reach the equilibrium dihedral angle. The dihedral angle (θ) is defined (**a**) as $\gamma_{ss} = 2\gamma_{sf} \cos(\theta/2)$, where γ_{ss} and γ_{sf} are the solid–solid and the solid–fluid interfacial energies, respectively⁹. A value for θ of $<60^\circ$ indicates that, even at small fluid fractions, the fluid fills channels between grains and efficient percolation is possible. When $\theta > 60^\circ$, the fluid will accumulate into disconnected pockets if the fluid fraction is low, and in this case complete percolation is not possible. Thus, 60° is the critical value for fluid permeability.

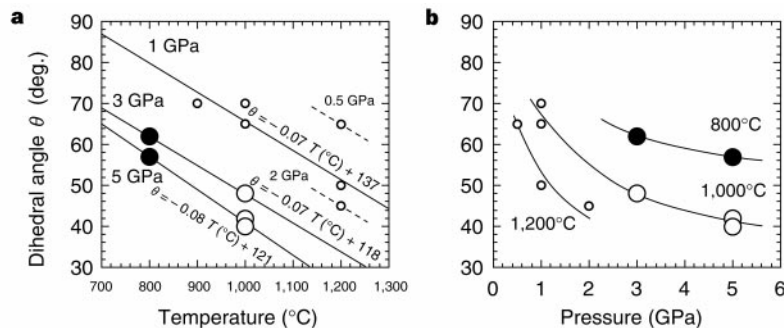


Figure 3 The aqueous fluid–forsterite–forsterite dihedral angle as a function of temperature and pressure. **a**, Temperature; **b**, pressure. The solid lines in **b** do not represent a fit to the data but are merely intended as a guide to the eye. The 800°C and

The observed reduction in dihedral angle with increasing pressure is attributed to compositional change in the fluid with pressure¹⁰. The magnitude of $(\partial\theta/\partial P)_T$ between 1 and 3 GPa is greater than that between 3 and 5 GPa (Fig. 3). This observation is consistent with the finding, that the solubility of silicate in aqueous fluid and the Mg/Si ratio of the fluid increase significantly above 3 GPa (refs 16–18). Although the temperature dependence of solubility at these high pressures has not been reported, experiments at lower pressures¹⁹ indicate that the solubility of silicate in aqueous fluid increases with increasing temperature. If this trend is maintained at higher pressures, the observed reduction in dihedral angle with increasing temperature may also be attributed to the solubility change.

It is often suggested that the generation of most subduction zone magmas is initiated by fluid-triggered melting of the mantle wedge above slabs of subducting oceanic lithosphere^{1–8}. It has also been suggested^{3–8} that the mantle wedge becomes hydrated by H_2O released by dehydration of the slab at relatively shallow depths, and that this peridotite may carry H_2O beneath the volcanic arc. The thickness of this downdragged hydrous peridotite (DHP) is estimated to be less than that of the oceanic crust⁷, or about 1–3 km (ref. 8), based on the stability fields of hydrous minerals and temperature structure in the mantle wedge.

By combining our data with previous results^{10,11}, we have calculated the pressure–temperature (P – T) trajectory of the 60° dihedral angle isopleth (Fig. 4). A possible P – T range of the upper surface of the DHP^{20,21} is also shown in Fig. 4. From these data and calculations (Fig. 4) it is evident that most of the aqueous fluid generated by the successive decomposition of hydrous minerals in DHP cannot segregate from solids and will be transported to greater depths as free fluid in isolated pores among crystals, owing to the large dihedral angle ($\theta > 60^\circ$) at low pressures and temperatures. The P – T trajectory of the DHP intersects the 60° dihedral angle isopleth at pressures in the 3–5 GPa range (about 80–150 km depth). Because the 60° dihedral angle isopleth crosses the DHP obliquely (Fig. 5), aqueous fluid will be supplied continuously from the DHP at any depth greater than the point of intersection.

These experimental results have important implications for models explaining the origin and position of the volcanic front in arcs. The volcanic front (VF), defined as the trenchward boundary of stratovolcanoes in subduction zones²², generally forms a well defined line parallel to the trench axis. No magmatism is observed on the trench side of the VF. A characteristic tectonic feature of the VF is its position 80–150 km above the Wadati–Benioff zone²³.

Several mechanisms have been proposed to explain the origin of the VF. The formation of the VF has been related to the stability limit of amphibole in the mantle wedge^{4,6}. From numerical simulations⁷, it has been suggested that the VF represents limitations on melt movement and that these limitations result from restrictions imposed by solid state convection within the peridotite

$1,200^\circ\text{C}$ curves in **b** are drawn to be consistent with the shape of the $1,000^\circ\text{C}$ curve. Large solid circle: this study. Large open circle: data from ref. 11. Small open circle: data from ref. 10.

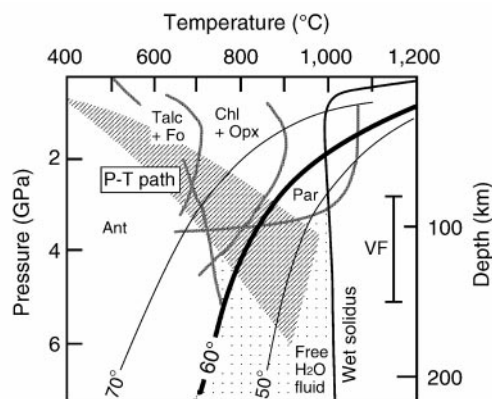


Figure 4 The 60° isopleth of the dihedral angle in aqueous fluid–forsterite–forsterite in P – T space. The phase relation in the system peridotite– H_2O ²⁴ is also shown. The downdragged hydrous peridotite (DHP) would be formed at the base of the mantle wedge by the H_2O supplied from the oceanic crust^{3–8}. The mechanisms of this migration of H_2O from the oceanic crust to the slab/mantle interface has not yet been clarified, but possible mechanisms include: (1) dihedral angle may also become <60° at higher pressure for minerals which constitute the oceanic crust, although water is non-wetting against those minerals at low pressure³⁰; (2) fluid migrates by hydrofracturing³¹; or (3) fluid rises as a diapir within the oceanic crust as a result of inherent buoyancy. The possible P – T path^{20,21} of the upper surface of DHP is within the shaded region denoted P – T path. The thickness of DHP is set to be 3 km as an example. Note that the P – T path of the upper surface of DHP intersects the isopleth at depths corresponding to the top of the deep seismic zone (Wadati–Benioff zone) just beneath the volcanic front (range bar denoted VF). The uncertainty in the point at which the 60° isopleth intersects the P – T path of DHP is ± 0.5 GPa, which corresponds to a ± 2 km in the DHP thickness. Because olivine is anisotropic and the actual mantle is a polyphase mixture rather than pure olivine, an uncertainty of $\sim \pm 10^\circ$ in the 60° isopleth is estimated. The shift of the intersections by these effects is indicated by the 50° and 70° isopleths. Abbreviations: Fo, forsterite; Opx, orthopyroxene; Par, pargasite; Chl, chlorite; Ant, antigorite.

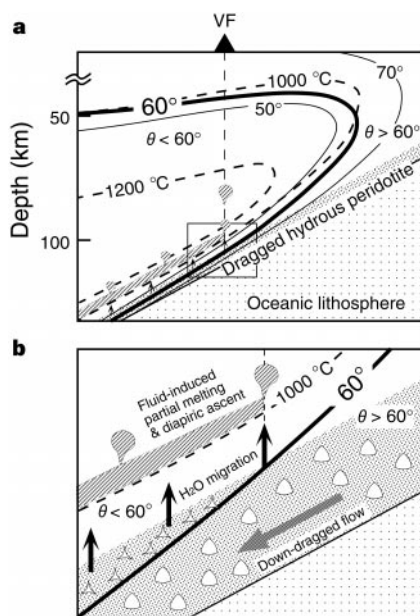


Figure 5 Model distributions of temperature and dihedral angles at a convergent margin. **a**, Overview. **b**, Enlargement of the central part of **a**. Open symbols with concave and convex surface indicate the geometry of grain-boundary aqueous fluid for dihedral angles of <60° and >60°, respectively. To illustrate our scenario more clearly, the temperature structure and the thickness of DHP are expressed using average values. Uncertainty estimates allow minimum and maximum depths of water release of 60 and 180 km, respectively, as discussed in the legend to Fig. 4. Note that migration of trapped aqueous fluid becomes possible in the region where dihedral angle is <60°.

mantle wedge. These studies make the *a priori* assumption that unrestricted fluid flow occurs in the mantle wedge, whereas only ref. 5 explicitly considers the effect of the fluid-wetting properties on mantle permeability.

Our results lead to an alternative explanation for the position of the VF in island arcs. The coincidence of the release depth of aqueous fluid from DHP and the position of the VF suggests that the location of the VF is controlled by the fluid connectivity in the mantle wedge. When the aqueous fluid migrates upwards and into the warmer mantle wedge and the solidus temperature of peridotite under water-saturated conditions is reached²⁴, partial melting of the mantle-wedge peridotite occurs (Fig. 5). Because partially molten peridotite is less dense than the surrounding mantle material, partially molten peridotite then ascends as a mantle diapir²⁵ (Fig. 5). Thus, magmatism beneath the VF is initiated in the region where aqueous fluid is supplied from the DHP.

The continuous supply of aqueous fluid behind the VF, resulting from the oblique intersection of the 60° dihedral angle isopleth with the pressure–temperature coordinates of the DHP (Fig. 5), may lead to multiple volcanic chains rather than a single chain in the volcanic arc. This is consistent with the fact that most volcanic arcs can be wide (up to 180 km)⁶, with observed across-arc variation in trace element and isotopic compositions²⁶. This mechanism for H_2O release in the peridotite mantle wedge may explain why the VF overlies the Wadati–Benioff zone at depths ranging from 80 to 150 km (ref. 23) in most subduction zones, even though the decomposition of hydrous minerals in the oceanic lithosphere and the DHP may occur at depths between 40 and 300 km (refs 8, 27). □

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A dromaeosaurid dinosaur with a filamentous integument from the Yixian Formation of China

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Dromaeosaurids, despite their notoriety, are poorly characterized meat-eating dinosaurs, and were previously known only from disarticulated or fragmentary specimens¹. Many studies have denied their close relationship to birds^{2,3}. Here we report the best represented and probably the earliest dromaeosaurid yet discovered, *Sinornithosaurus millenii* gen. et sp. nov., from Sihetun, the famous Mesozoic fish–dinosaur–bird locality in China^{4,5}. *Sinornithosaurus* not only greatly increases our knowledge of Dromaeosauridae but also provides evidence for a filamentous integument in this group. It is remarkably similar to early birds postcranially. The shoulder girdle shows that terrestrial dromaeosaurids had attained the prerequisites for powered, flapping flight⁶, supporting the idea that bird flight originated from the ground up^{7,8}. The discovery of *Sinornithosaurus* widens the distribution of integumentary filaments among non-avian theropods^{5,9,10}. Phylogenetic analysis indicates that, among known theropods with integumentary filaments or feathers^{2,5}, Dromaeosauridae is the most bird-like, and is more closely related to birds than is Troodontidae.

Theropoda Marsh 1881

Maniraptora Gauthier 1986

Dromaeosauridae Matthew & Brown 1922

Sinornithosaurus millenii gen. et sp. nov.

Etymology. '*Sinornithosaurus*' derived from Sino-Ornitho-Saurus, meaning a bird-like dinosaur from China; '*millenii*' derived from Millennium, Latin for one-thousand years, referring to its discovery near the end of the twentieth century.

Holotype. IVPP (Institute of Vertebrate Paleontology and Paleoanthropology) V12811 (Figs 1, 2).

Locality and horizon. Sihetun, western Liaoning, China. Layer 6, lower (Chaomidianzi²) Yixian Formation⁴, Jehol Group; probably Early Cretaceous¹¹.

Diagnosis. Differing from other dromaeosaurids in the presence of ornament-like pits and ridges on the anterolateral surface of the antorbital fossa; the posterolateral process of the parietal turning sharply posteriorly; the dentary bifurcated posteriorly; unserrated premaxillary teeth; supracoracoid fenestra of coracoid; manual phalanx III-1 more than twice the length of phalanx III-2; pronounced tubercle near the midshaft of the pubis; posterodorsal process of the ischium; partially arcotometatarsalian metatarsal III.

Description. *Sinornithosaurus* is a small dromaeosaurid, with a skull about 13 cm long (Figs 2, 3, Table 1). It has a relatively small antorbital fenestra and a large orbit, as in other dromaeosaurids. The antorbital fossa, anterior to the antorbital fenestra, is large and well-demarcated, although its posterodorsal margin is obscured by surface damage. The anterolateral surface of the antorbital fossa bears a number of pits and ridges. The latter are well-marked and show no trace of pathological origin. Two small maxillary fenestrae, normally seen in dromaeosaurids and some other theropods, lie in the antorbital fossa. The exposure of the frontal is extensive and is more than twice the length of the parietal. The prefrontal is sutured with the lacrimal as in *Deinonychus*¹². The frontal process of the triradiate postorbital is not upturned, unlike those in other dromaeosaurids¹³. The parietals form a narrow sagittal crest, as in many non-avian theropods and birds. *Sinornithosaurus* is placed in Dromaeosauridae on the basis of the following shared derived features^{13–15}: T-shaped lacrimal; large supratemporal fossa with a strongly sinusoidally curved anterior frontal margin; T-shaped quadratojugal (although the vertical bar is relatively shorter); widely open fenestra between the quadratojugal and quadrate; dentary with subparallel dorsal and ventral margins; ossified caudal rods increasing the lengths of prezygapophyses and chevrons (for more details, see Supplementary Information).

The shoulder girdle and forelimb of *Sinornithosaurus* closely resemble those of early birds (Fig. 4a–d). As in *Archaeopteryx*^{16,17}, the articulated left scapula and left coracoid form an angle of less than 90°, and the scapula is shorter than the humerus (0.63) and ulna (0.77), and forms most of the laterally facing glenoid. The anterolateral part of the right coracoid of *Sinornithosaurus* is distorted by lateromedial folding (Fig. 4c), and was broader in life and differed little from those of other dromaeosaurids, such as *Deinonychus*¹⁸ and an undescribed specimen of *Saurornitholestes* (TMP (Royal Tyrrell Museum of Palaeontology) 88.121.39), except for the presence of a supracoracoid fenestra. The coracoid bears a pronounced biceps tubercle and its lateral profile is almost identical to that of *Archaeopteryx*¹⁹. The furcula, half of which is extensively

Table 1 Lengths of selected elements of *Sinornithosaurus millenii* (IVPP V12811)

Skull	130*
Mandible	125
Left scapula	85
Right coracoid	44
Right sternum	84*
Posterior two dorsal vertebrae	26
Five sacral vertebrae	65
Right humerus	134
Right ulna	110
Metacarpal II	63
Three phalanges of manual digit II	89
Left ilium	85
Left pubis	116
Left ischium (along posterior edge)	52
Left femur	148*
Right tibia (preserved length)	125
Metatarsal III (longest)	93
Four phalanges of foot digit III	73

* Estimation. Length measurements are given in millimetres.

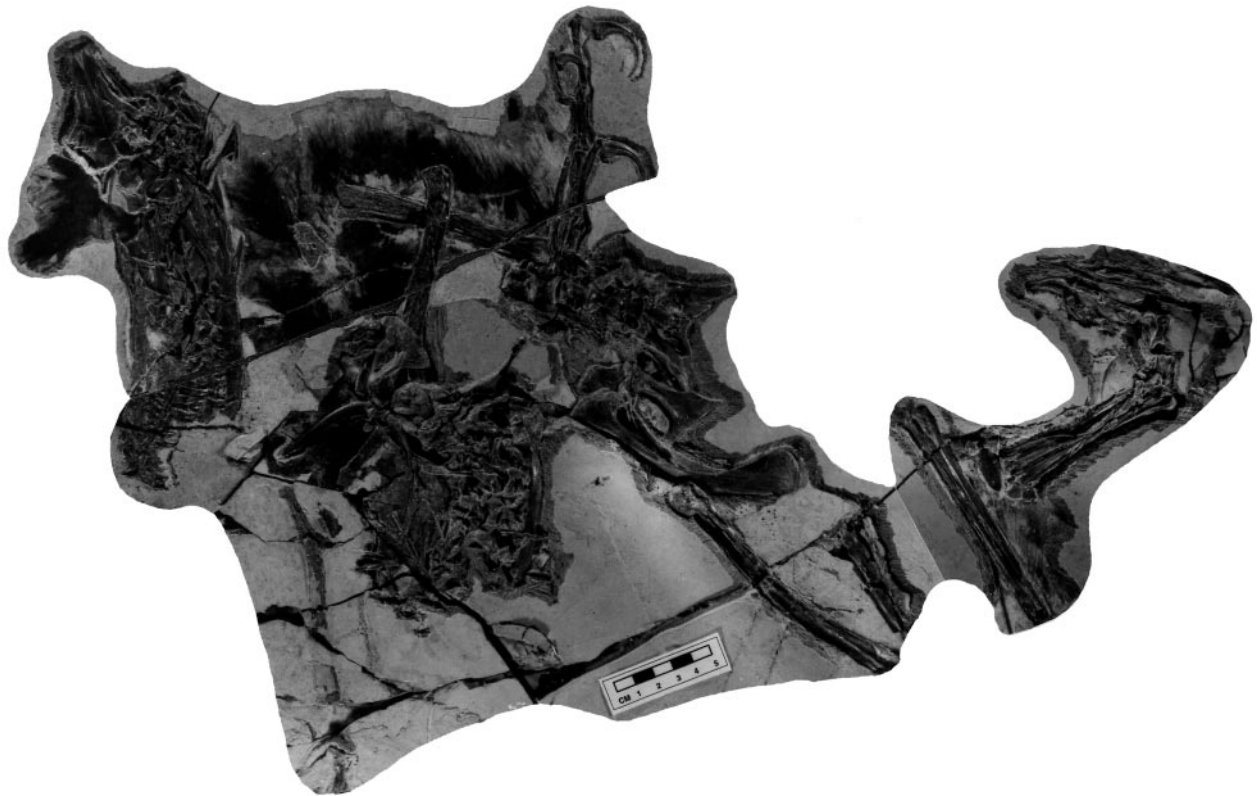


Figure 1 *S. millenii*, Holotype, IVPP V12811. The posterior part of the skull is disarticulated from the snout and lower jaws and turned 180° in the opposite direction. The postcranial skeleton is also not articulated, but the bones retain a close association.

Integumentary filaments have been displaced, lacking their direct relationships to bony elements.

covered by other elements, gently curves in a 'boomerang'-shaped configuration (Fig. 2), differing from that of *Velociraptor*²⁰ but resembling that of *Archaeopteryx*¹⁶. Its shaft is compressed and tapers distally. A pair of ossified sterna appear similar to those of other dromaeosaurids²¹, including *Saurornitholestes* (TMP 92.36.333). The length of each sternum is slightly more than twice its width, and its constricted anterolateral side bears a series of possibly five rib attachments (Fig. 2). These costal facets on the sternum imply the presence of hinged sternocostal joints in dromaeosaurids, which is not concordant with recent arguments about the ribcage-pectoral girdle complex and the respiratory pattern of theropods²². As in other dromaeosaurids and birds^{1,21}, the coracoid facet of the sternum faces much more anteriorly than laterally. The forelimb has the greatest relative length of those known among non-avian theropods, and it is estimated to be about 80% of the length of the hindlimb. This is also indicated by the ratios of the ulna to the scapula (1.29) and the ulna to metatarsal III (1.18). These ratios are less than 1.0 in most other non-avian theropods. Metacarpal III is bowed laterally, as in other dromaeosaurids and birds³.

Sinornithosaurus is also more bird-like than other non-avian theropods in the following derived features of the pelvic girdle and hindlimb (Fig. 4e, f): pubic peduncle of ilium broader than acetabulum³; open acetabulum tending to close off medially^{8,23}; posteroventrally directed pubis bearing a short symphysis (less than half the length of the bone), and its distal end cup-like¹; short ischium plate-like and less than half the length of the pubis, indicating, as in *Velociraptor*¹, *Unenlagia*⁸ and birds³, the absence of ischial symphysis; thin fibular shaft about 1/7 of the diameter of the tibia³.

It has been stated that the forelimbs of dromaeosaurids could not move like those of *Archaeopteryx* because of the posteroventrally facing glenoids^{8,24}. This assumption is based on *Deinonychus*¹⁸,

the scapula and coracoid of which are actually incomplete. We believe that the glenoid was formed primarily by the scapula in *Deinonychus*, judging from the glenoid part of the coracoid, and may have faced laterally as in *Sinornithosaurus*, *Velociraptor*¹ and *Saurornitholestes* (TMP 88.121.39). That the coracoid and scapula form a sharp angle, as in *Sinornithosaurus*, may also be true for other dromaeosaurids. The shoulder girdle, as exemplified in *Sinornithosaurus*, is similar to that of *Archaeopteryx*¹⁹. A laterally facing glenoid is consistent with an avian mode of movement (elevation and relevant rotation and abduction) of the forelimb^{8,24}. The modifications of the scapulocoracoid and coracosternal articulations in Dromaeosauridae altered the orientation of the shoulder girdle, perhaps enabling a wider, more avian range of motion at the glenohumeral joint. Because *Sinornithosaurus* and other dromaeosaurids were bipedal, cursorial terrestrial dinosaurs and are closely related to birds (see below), the anatomical modification of their shoulder girdles supports a cursorial origin for avian flight^{6,7}.

The body of *Sinornithosaurus* was apparently covered by a layer of integumentary filaments (Figs 1, 2). These filaments are not in their original positions, owing to posthumous displacement, but are distributed as patches underneath or close to most bony elements, including the skull. They have been exposed by preparation only in a small part of the specimen and have sometimes been cut off near the edges of elements that needed to be exposed (see Fig. 1). The anatomical structure of these filaments is not discernible, but in appearance they differ little from the external filaments of other theropod dinosaurs^{2,5,10} or even the plumulaceous feathers of *Confuciusornis* (IVPP V11307) from the same locality. The filaments generally reach 40 mm in length. Those near the postcranial elements seem longer than those around the skull. The filaments around the tibia are relatively sparse. It is uncertain whether *Sinornithosaurus* had, in life, rectrix-like structures, as seen in both *Caudipteryx* and *Protarchaeopteryx*, or remix-like

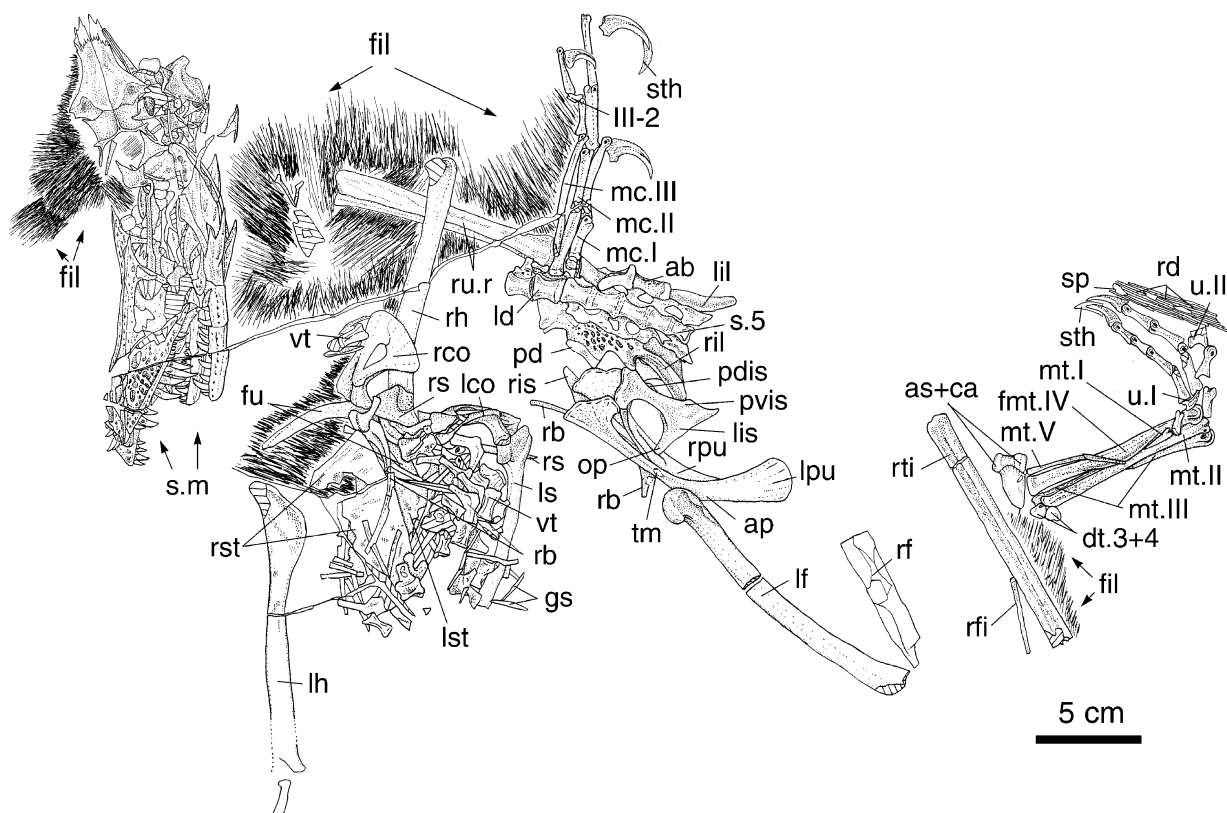


Figure 2 *S. millenii*. Drawing of the specimen shown in Fig. 1; the specimen represents an adult individual, as indicated by the five co-ossified sacral vertebrae and the partially fused astragalus and calcaneum. Abbreviations: ab, acetabulum of ilium; ap, apron of pubis; as + ca, astragalus and calcaneum; dt.3 + 4, distal tarsals 3 + 4; fil, integumentary filaments; fmt.IV, enlarged ventral flange of metatarsal IV; fu, furcula; gs, gastralia; lco, left coracoid; ld, last dorsal vertebra; lf, left femur; lh, left humerus in anteromedial view; l.il, left ilium; lpu, left pubis; lis, left ischium; ls, left scapula; lst, left sternum; mc.I-III, metacarpals I-III; mt.I-V, metatarsals I-V; op, obturator process; pd,

pubic peduncle of ilium; pdis, posterodorsal process of ischium; pvis, posteroventral process of ischium; rb, rib; rco, right coracoid; rd, elongated prezygapophyses and chevrons of mid- and posterior caudal vertebrae; rf, right femur; rfi, right fibula; rh, right humerus; ril, right ilium; ris, right ischium; rpu, right pubis; rs, right scapula; rst, right sternum in ventral view; rti, right tibia; ru.r, right ulna and radius; s.m, skull and mandible; sp, spine of caudal vertebra; s.th, horny sheath; s.5, sacral vertebra 5 (the last); tm, tubercle for muscle attachment; u.I, ungual of digit I of pes; u.II, enlarged, slashing ungual of digit II of pes; vt, vertebra; III-2, phalanx 2 of digit III of manus.

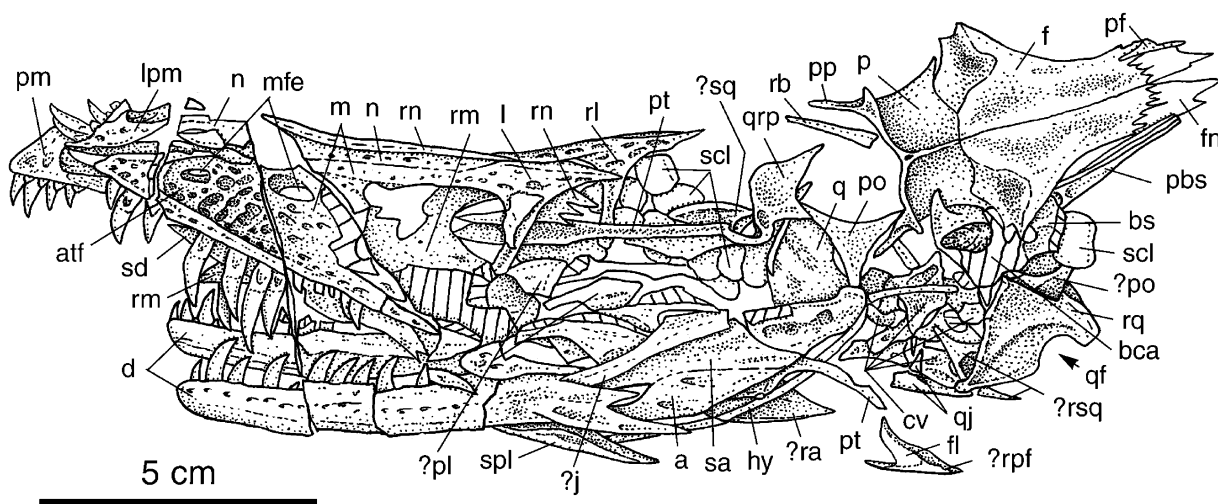


Figure 3 *S. millenii*, skull with lower jaws. Abbreviations: a, angular; atf, antorbital fossa; bca, braincase; bs, basisphenoid; cv, elements of the disarticulated first and second cervical vertebrae; d, dentary; f, frontal; fl, facet for lacrimal; fn, facet for nasal; hy, hyoid; l, lacrimal; lpm, left premaxilla in medial view; m, maxilla; mfe, maxillary fenestrae; n, nasal; p, parietal; pbs, parabasisphenoid process; pf, left prefrontal (the anterolateral part overlapped by the lacrimal was not preserved); pm, right premaxilla in medial view; po, left postorbital; pp, posterolateral process of the parietal; pt, pterygoid; q, quadrate; qf,

fenestra between quadratojugal and quadrate; qj, quadratojugal; qrp, quadrate ramus of pterygoid; rb, rib; rl, right lacrimal in medial view; rm, right maxilla in medial view; rn, right nasal; rq, right quadrate in anteromedial view; sa, surangular; scl, sclerotic bones; sd, supradentary bone; spl, splenial; ?j, left jugal; ?pl, palatine; ?po, right postorbital; ?rpf, right prefrontal; ?rsq, right squamosal in ventral view; ?sq, left squamosal; ?ra, right angular. Most elements of the palate and braincase are obscured by cracks and dislocation.

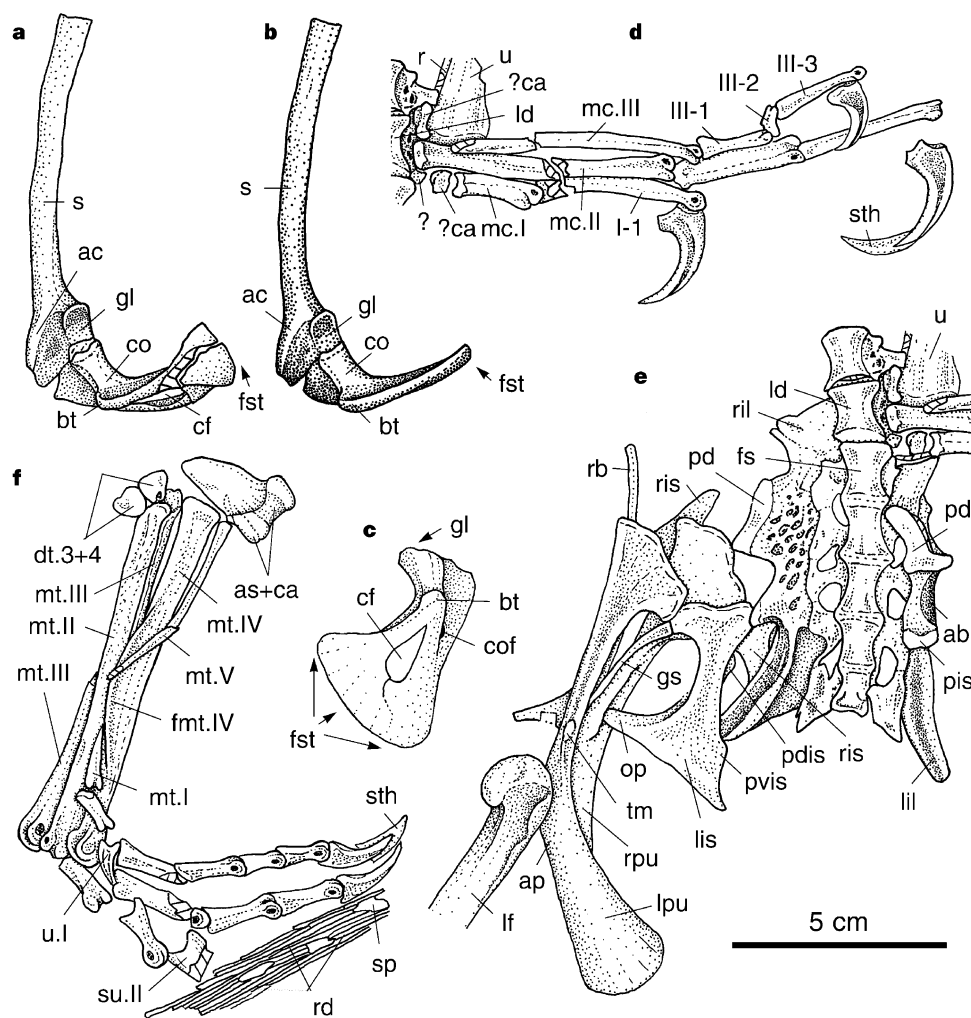


Figure 4 *S. millenii*, selected elements of the postcranial skeleton. **a, b**, Left scapula and coracoid in lateral view (original, **a**; reconstructed, **b**). **c**, Right coracoid in ventral view, with its anteromedial portion slightly folded. **d**, Right manus mainly in posteroventral view. **e**, Sacrum (in ventral view) and pelvic girdles. **f**, Tarsals, right pes (mainly in posteroventral view) and partial tail. Abbreviations: ac, acromion; bt, biceps tubercle; cf,

supracoracoid fenestra; co, coracoid; cof, coracoid foramen; ?ca, ?carpal; fst, facet for sternum; fs, first sacral vertebrae; gl, glenoid of shoulder girdle; pis, ischial peduncle of ilium; r, radius; s, scapula; su.ii, slashing ungual of digit II of pes; u, ulna; I-1, phalanx 1 of digit I of manus; III-1, III-2, III-3, phalanges 1-3 of digit III of manus; u, ulna; ?, possibly a carpal. For other abbreviations see Fig. 2.

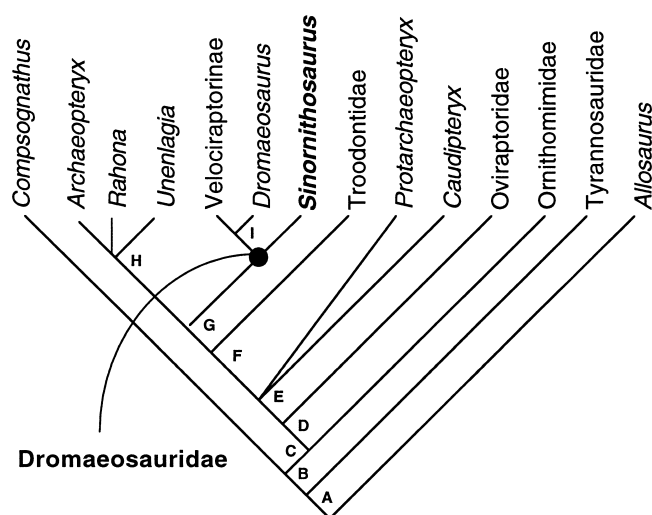


Figure 5 Cladogram showing phylogenetic relationships of Dromaeosauridae (including *Sinornithosaurus*) within the derived theropods. This is a strict consensus tree of our four most parsimonious trees based on 106 characters and 14 taxa (tree length = 183; consistency index = 0.639; retention index = 0.697). Multistate characters are unordered; tree produced using the branch-and-bound option of PAUP³⁰. Synapomorphies for each node were determined by acceleration transformation (ACCTRAN). *Sinornithosaurus* is the basal member of Dromaeosauridae, which can be defined mainly by the following six unambiguous synapomorphies: characters 86 (lacrima T-shaped); 88 (supratemporal fossa covering most of frontal process of postorbital and extending anteriorly on dorsal surface of frontal to at least level of posterior orbital margin); 90 (quadratojugal Y- or T-shaped); 91 (quadratojugal fenestra widely open); 96 (in lateral view upper and ventral margins of dentary subparallel); and 100 (ossified caudal rods extending lengths of prezygapophyses and chevrons). The close relationship of Dromaeosauridae to birds is primarily based on 10 unambiguous synapomorphies (node G): characters 39 (body of coracoid forming sharp angle with body of scapula); 46 (metacarpal III bowed laterally); 59 (pubic shaft projecting posteroventrally relative to long axis of sacral vertebrae); 65 (shape of ischial shaft mediolaterally compressed and plate-like along entire length); 104 (ulna longer than metatarsal III); 105 (glenoid of pectoral girdle facing laterally); 106 (articular facet of coracoid on sternum almost anterior); and the other three (characters 15, 92 and 93) of the 10 synapomorphies are unknown in *Sinornithosaurus*. For the list of the synapomorphies of the other clades see Supplementary Information.

structures, as seen attached to the arm of *Caudipteryx*, because evidence of the direct relationship of the preserved filaments to the relevant bones is obscured by preservation. The broad distribution of the filaments across the body of *Sinornithosaurus* further demonstrates that the clusters of fine filaments in *Sinosauropteryx*⁵ do not represent internal collagenous fibres for skin support in semi-aquatic animals²⁵, but integumentary derivations of terrestrial animals. *Sinornithosaurus* is the fifth kind of theropod known to possess integumentary filaments. It is possible that these filaments indicate an insulatory layer that served to maintain body heat².

It has been proposed that birds are descendants of derived theropods^{6,16,26}, but there has been no consensus as to which theropod group is most closely related to birds. Dromaeosauridae^{27,28}, Troodontidae^{3,29} and *Caudipteryx*² have all recently been suggested to be closely related to birds, but previous studies have not considered all of these taxa simultaneously, making their results arguable.

We investigated the phylogenetic position of *Sinornithosaurus* using a data matrix of 106 characters and 14 taxa^{3,13} (see Supplementary Information). We included all of the theropod taxa that have been proposed to be closely related to birds^{2,3,8}. As with a previous study², we did not include characters relative to integumentary filaments and feathers because we cannot determine whether their absence in most other derived theropods is real or merely an artefact of preservation. Our analysis indicates that *Sinornithosaurus* is a basal dromaeosaurid and strongly supports the view that Dromaeosauridae and birds (where Aves is Avialae²⁶) are more closely related to each other than either is to Troodontidae (Fig. 5, node G). Our analysis also indicates that *Protarchaeopteryx* and *Caudipteryx* are more remote from birds than is Troodontidae. We re-analysed the data matrix by sequentially adding steps to test the robustness of the relationships between the taxa. The close relationship between Dromaeosauridae and birds is the most robust and did not collapse until five more steps had been added. The currently established phylogenetic relationships among derived theropods seem to support the presence of true feathers in Dromaeosauridae, but the validity of this interpretation cannot be confirmed until more direct evidence is available. □

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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Methane formation from long-chain alkanes by anaerobic microorganisms

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Biological formation of methane is the terminal process of biomass degradation in aquatic habitats where oxygen, nitrate, ferric iron and sulphate have been depleted as electron acceptors. The pathway leading from dead biomass to methane through the metabolism of anaerobic bacteria and archaea is well understood for easily degradable biomolecules such as carbohydrates, proteins and lipids^{1,2}. However, little is known about the organic compounds that lead to methane in old anoxic sediments where easily degradable biomolecules are no longer available. One class of naturally formed long-lived compounds in such sediments is the saturated hydrocarbons (alkanes)^{3–5}. Alkanes are usually considered to be inert in the absence of oxygen, nitrate or sulphate⁶, and the analysis of alkane patterns is often used for biogeochemical characterization of sediments^{7,8}. However, alkanes might be consumed in anoxic sediments below the zone of sulphate reduction^{9,10}, but the underlying process has not been elucidated. Here we used enrichment cultures to show that the biological conversion of long-chain alkanes to the simplest hydrocarbon, methane, is possible under strictly anoxic conditions.

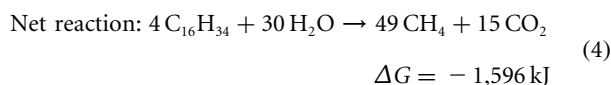
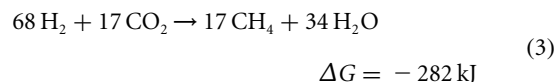
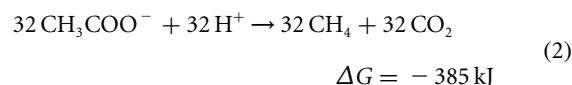
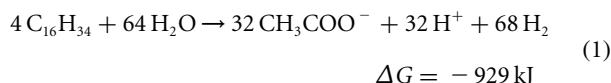
To study methane formation (methanogenesis) from long-chain alkanes, hexadecane ($n\text{-C}_{16}\text{H}_{34}$), a representative of this class of

compound, was incubated under strictly anoxic conditions in sulphate-free medium inoculated with anoxic ditch sediment. After four months, gas formation in the presence of hexadecane increased steadily in comparison with an inoculated control without hexadecane. By repeated transfer of aliquots to new media, we obtained a sediment-free enrichment culture which formed gas in the presence of hexadecane as substrate (Fig. 1). The gas was mostly methane. Methane production in freshly inoculated cultures was favoured when low concentrations of sulphate (≤ 2 mM) were added. This stimulatory effect was probably due to the resulting background growth of sulphate-reducing bacteria that favoured growth conditions for anaerobic microorganisms involved in methanogenesis. Microscopic examination of the sediment-free enrichment culture revealed oval, hair-like and rod-shaped microorganisms, which mainly occurred in loose aggregates, and vibrioid forms homogeneously distributed in the medium. Methane formation and growth of microorganisms also occurred with pentadecane, but not with decane or hexane.

In a study of methanogenesis in oil wells, it was thought that aerobic hydrocarbon-degrading bacteria excrete polar oxidation products (for example, fatty acids) that may be subsequently consumed by anaerobic methanogenic communities¹¹. However, in the case of our enrichment culture we could exclude the possibility that aerobic bacteria initiated alkane degradation by traces of oxygen that might diffuse slowly through the stoppers. No growth occurred when aliquots were incubated with hexadecane under atmospheric or lower oxygen partial pressure, indicating the absence of aerobic or microaerobic alkane-utilizing microorganisms. Furthermore, methane-forming enrichment cultures with hexadecane grew equally well when the butyl rubber-sealed bottles were incubated inside an anoxic jar containing pure nitrogen and an oxygen trap.

Fatty-acid analysis revealed only acetate at low concentration (7.5 μ M) in the enrichment culture. Further accumulation during growth was not observed. With repeated release of overpressure during growth, methane and carbon dioxide became the dominant gases in the culture head space (approximate molar ratio 15:2). Ethane or other gaseous hydrocarbons were not detected. Hydrogen was only detectable at trace concentrations (partial pressure 1.7 Pa). Low concentrations of acetate and hydrogen agree with their proposed role as key intermediates during methanogenesis from natural organic compounds^{1,2}. Decomposition of such compounds by anaerobic bacteria produces acetate and hydrogen, which are consumed by methanogenic archaea and hence maintained at very low concentrations. Conversion of hexadecane to CH_4 and CO_2 is expected to involve at least three groups of microorganisms, analogous to methane formation from other molecules with more than two carbon atoms (for example, propionate and higher fatty acids)^{1,2}. These microorganisms are assumed to be acetogenic (syntrophic)

bacteria decomposing hexadecane to acetate and H_2 (equation (1)), a group of archaea cleaving acetate into CH_4 and CO_2 (equation (2)), and another group of archaea converting CO_2 and H_2 to CH_4 (equation (3)).



Free-energy changes in kJ are shown for the given stoichiometries in mol; they were calculated for the given concentrations determined in the enrichment culture (at pH 7.0). All partial reactions (equations (1–3)) are thermodynamically feasible (exergonic). The acetogenic reaction (equation (1)) would not be possible (endergonic) at standard activities of acetate and H_2 . The free-energy change per mol of CH_4 in the net reaction is -32.6 kJ.

For experimental proof of the postulated net reaction (equation (4)), we determined the degradation balance by quantifying the remaining hexadecane and methane in an enrichment culture. Out of 1.7 mmol hexadecane added initially, 0.59 mmol were consumed during the formation of 4.6 mmol CH_4 (summed from analysed gas samples taken anoxically during 810 days of incubation). Theoretically (equation (4)), the consumed amount of hexadecane would yield 7.2 mmol CH_4 . The deviation can be explained by partial utilization of organic carbon and reducing equivalents from hexadecane for cell synthesis in the microorganisms, and by a small loss of methane during the repeated sampling procedures.

Conversion of consumed hexadecane to CH_4 and CO_2 was verified in subsequent growth experiments with ^{13}C -labelled substrate. Pure ^{13}C -hexadecane in the enrichment culture retarded growth significantly; therefore, a 1/9 (vol./vol.) mixture of ^{13}C -labelled and unlabelled hexadecane was applied. The ^{13}C -content in CH_4 and CO_2 after 158 days of incubation was 10.1 (± 0.8) and 1.85 (± 0.001) atom%, respectively. In a control experiment using unlabelled hexadecane, the ^{13}C -content in CH_4 and CO_2 was 1.07 and 1.09 atom%, respectively.

Both quantitative experiments confirm that hexadecane was converted to methane. The high ^{13}C -content of methane in the labelling experiment is in accordance with methane formation mainly by acetate cleavage as in equation (2). Methane produced by this reaction alone should have approximately the same ^{13}C -content as the applied hexadecane, which was 11.0 atom% (calculated from mixing ratio); however, this highly ^{13}C -enriched methane mixes with methane with a lower ^{13}C -content originating from CO_2 from the bicarbonate buffer (equation (3)). This buffer was initially unlabelled and became only gradually enriched by $^{13}\text{CO}_2$ from acetate cleavage (equation (2)). An exact calculation of the proportion of methane formed by CO_2 reduction during this experiment is not possible because of a background sulphate concentration and a background growth of sulphate-reducing bacteria (*Desulfovibrio*; see below). These bacteria probably consumed part of the hydrogen formed by acetogenic bacteria (equation (1)). Hence, significantly less than one third of total methane was probably formed by CO_2 reduction during the incubation period.

We tested for microorganisms that may catalyse the reactions in equations (1–3) by screening 30 bacterial and 30 archaeal 16S

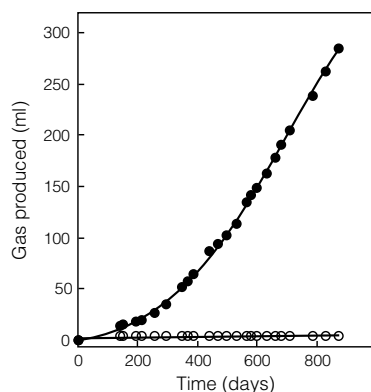


Figure 1 Formation of gas in an anaerobic enrichment culture growing in 100 mineral medium with hexadecane (filled circles) as the only organic substrate. An inoculated parallel control without hexadecane (open circles) did not show gas formation.

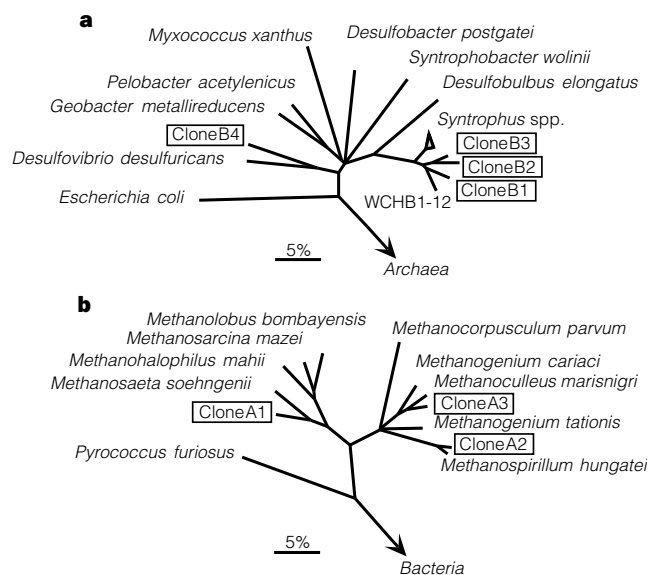


Figure 2 Reconstructed phylogenetic trees based on 16S rRNA sequences that were retrieved from the methanogenic enrichment culture on hexadecane. **a**, Relationships of bacterial clones (B1–B4) within the delta subclass of *Proteobacteria*. **b**, Relationships of archaeal clones (A1–A3) within methanogenic *Archaea*. Bars indicate 5% estimated

sequence divergence. Group designated as *Syntrophus* spp. comprises sequences of species *Syntrophus gentianae*, *Syntrophus buswellii* and *Syntrophus acidotrophicus*; the latter and clone WCHB1-12 from a solvent-contaminated aquifer²⁹ were the closest relatives of the retrieved clones (sequence similarities around 95%).

ribosomal RNA gene clones retrieved from the enrichment culture. Four distinct restriction patterns were observed among the bacterial clones, and three patterns among the archaeal clones. Representative clones of each pattern were chosen for sequencing followed by phylogenetic reconstruction¹². All bacterial clone types were affiliated with the delta subclass of *Proteobacteria* (Fig. 2a). Three of them (representing 26 screened clones) were closely related to cultured syntrophic bacteria of the genus *Syntrophus*. One bacterial clone type was affiliated to *Desulfovibrio*, a genus that comprises incompletely oxidizing, H₂-utilizing sulphate-reducing bacteria¹³. All archaeal clone types were affiliated to methanogenic *Archaea* (Fig. 2b). One clone type was most closely related to the genus *Methanosaeta*, which comprises acetoclastic methanogens. Two clone types were related to the genera *Methanospirillum* and *Methanoculleus*, which comprise methanogens utilizing H₂ and CO₂. These results agree with microorganisms catalysing the reactions in equations (1–3). *Desulfovibrio* is probably present because of the low concentration of sulphate added to stimulate growth of the microbial community.

In conclusion, our results show that anaerobic bacterial communities can convert long-chain alkanes to methane, the simplest hydrocarbon. This net process may be regarded as 'microbial alkane cracking'. To our knowledge, such a biological process has not been demonstrated or studied by quantitative anoxic growth and labelling experiments. Some decades ago, observations with crude enrichment cultures containing oil fractions and calcium acetate as a supplementary substrate suggested that methane formation occurred from paraffin waxes¹⁴; however, individual alkanes were not studied and some control experiments were contradictory. The only hydrocarbons that were definitely shown to be degraded under conditions of methanogenesis were some monoaromatic compounds⁶ and alkenes^{15,16}. Alkanes contain only apolar sigma bonds and are, therefore, chemically even less reactive than aromatic hydrocarbons or alkenes. A few bacteria have been isolated that oxidize alkanes anaerobically; however, these require sulphate or nitrate as an electron acceptor⁶. The biochemistry of anaerobic alkane activation, which might involve the addition of a one-carbon compound, is only speculative so far⁶.

Demonstration of alkane conversion to methane and carbon dioxide has implications for the understanding of microbial processes during diagenesis. In many deep sediments, oxygen, nitrate,

ferric iron and sulphate are depleted, leaving methanogenesis as the only terminal degradation process^{1,17}. Because of diagenetic processes, including saturation of unsaturated aliphatic biomolecules, alkanes represent a significant fraction among the organic substances in aged sediments^{5,18}. It is therefore possible that alkanes are part of the hitherto largely unknown substrates that maintain slowly growing microbial communities and lead to methane formation in deep, old sediments^{17,19}. Bacterial communities as observed in the present enrichment culture may also contribute to the disappearance of alkanes and methane formation in coal-bed reservoirs where these processes have been partly attributed to bacterial activity²⁰. Bacterial methanogenesis from alkanes also appears, in principle, to be possible in organic-rich shales that show formation of relatively ¹³C-depleted ('biological') methane^{21,22}, and in crude oil reservoirs depleted of electron acceptors other than carbon dioxide. □

Methods

Growth of enrichment cultures

Enrichment cultures were grown in anoxic mineral medium buffered to pH 7 with bicarbonate (30 mM) plus CO₂ and reduced with sulphide (1 mM)¹³; sulphate was omitted in the initial culture; for subcultures, limited concentrations of sulphate were added (1–2 mM). Butyl rubber-sealed growth bottles contained an anoxic head space (one third or less of bottle volume) initially gassed with N₂/CO₂ (90/10, vol./vol.). Cultures were incubated at 28 °C. Overpressure due to microbial gas formation was intermittently released by insertion of a hypodermic needle connected to a volumetric device. The volume of the initial enrichment culture was 400 ml (including 150 ml ditch mud). Hexadecane was distributed by brief shaking which caused hydrocarbon droplets to associate with sediment particles. Hexadecane in sediment-free subcultures (100 ml) was provided on the surface of Teflon filter foil to allow a large contact area between the alkane and medium. The foil (pore size 0.45 µm) was autoclaved in a bottle under N₂ and then allowed to soak anoxically with a defined amount of hexadecane (0.5 ml; for labelling experiment 0.05 ml). The hexadecane-containing filter foil was fixed on the bottom by means of a glass rod and overlaid with anoxic mineral medium (100 ml). Decane and hexane for growth tests were diluted (1:3 and 1:50, respectively) in 2,2,4,4,6,8,8-heptamethylnonane as an inert carrier phase to avoid toxic effects of the shorter hydrocarbons. Anoxic jars for incubation of sealed culture bottles contained alkaline pyrogallol as oxygen trap. Aerobic growth tests were carried out in sulphide-free medium (20 ml; with sulphate) with hexadecane, and incubated in bottles (500 ml) under 1.0% and 20% O₂ (added to an N₂ atmosphere containing 10% CO₂).

Chemical analyses

Methane, carbon dioxide and hexadecane were measured by standard gas chromatographic methods. Trace concentrations of hydrogen were analysed by gas chromatography with a mercury oxide reduction detector²³. Fatty acids were converted to 2-nitrophenylhydrazides and analysed by high performance liquid chromatography²⁴. The ratio of

carbon isotopes was determined by mass spectroscopy after gas chromatographic separation (IRM-GC-MS)²⁵ using a DELTA XL instrument (Finnigan MAT).

Chemical synthesis

Labelled hexadecane was synthesized from uniformly ¹³C-labelled palmitic acid (Campro Scientific) by conversion to the methyl ester (with dimethyl sulphate), reduction to hexadecanol (with NaAlH₄), conversion to the *p*-tosylate ester, and reduction to the hydrocarbon (with NaAlH₄). After chromatographic separation, purity was confirmed by gas chromatography/mass spectrometry and NMR.

Nucleic-acid-based analyses

Sequences of 16S rRNA genes from the enrichment culture were amplified by PCR by using general primers for the domains *Bacteria* and *Archaea*. After cloning (pGEM-T Vector System II, Promega), inserts were screened by their restriction pattern (ARDRA) obtained with *Sau*3A. Almost full sequences from each restriction group were added to an alignment of about 13,000 homologous 16S rRNA primary structures by using the aligning tool of the ARB program package²⁶. Consensus trees were constructed after evaluation and correction according to distance matrix, maximum parsimony and maximum likelihood analyses of data sets¹². Ambiguous topologies are shown as multifurcations.

Calculation of free energy

Free-energy changes were calculated from ΔG_f° values²⁷. The used ΔG_f° value of hexadecane in the pure liquid state (+52.2 kJ mol⁻¹) as present in the culture was derived from the value for a (theoretical) gaseous standard state (+83.7 kJ mol⁻¹)²⁷ using the real vapour pressure (0.31 Pa) at 298 K. The latter was obtained by extrapolation from literature values²⁸ of vapour pressures (*p*) at other temperatures (*T*), using a plot of lg(*p*) versus 1/*T*.

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Correspondence and request for materials should be addressed to F.W. (e-mail: fwiddel@mpi-bremen.de). The nucleotide sequences of clones have been deposited at EMBL, Genbank under accession numbers AJ133791–AJ133793 (clones A1–A3) and AJ133794–AJ133797 (clones B1–B4).

Grouping of image fragments in primary visual cortex

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In the visual world, objects are partially occluded by nearer objects, separating them into image fragments. However, the image fragments of the object can easily be grouped and organized together by the visual system^{1–4}. Psychophysical data^{1–4} and theoretical analysis⁵ indicate that such perceptual grouping might be mediated in the early stages of visual processing. Here I show that some orientation-selective cells in the primary visual cortex (V1) have response properties that can mediate the grouping of image fragments. These cells stopped responding to a stimulus bar when it was partly occluded by a small patch. The cells also did not respond when the patch had uncrossed disparity so that it appeared to be behind the bar. However, the cells began responding again when the patch had crossed disparity so that it appeared to be in front of the bar. These results indicate that cells as early as V1 have the computational power to make inferences about the nature of partially invisible forms seen behind occluding structures.

Two line segments separated by a patch are often grouped together and perceived as a single vertical bar when the patch appears to be in front. However, the grouping of two image fragments becomes very difficult when the patch has uncrossed disparity so that it appears to be behind the image (Fig. 1a). Two Japanese monkeys were tested to see how they perceive a bar moving across patches of different height and disparity. Their judgements were similar and comparable to those of humans (Fig. 1b). Their judgements for a bar partially occluded by a patch of crossed disparity were comparable to those for the bar alone, and their judgements for a bar with a patch of uncrossed disparity were similar to those for two unconnected vertical segments. When the patch had zero disparity, so that it appeared to be at the same depth as the stimulus bar, the grouping of two unconnected line segments became more difficult with increasing patch height.

The responses of single cells in V1 to a bar with patches of different disparities were recorded. The responses of some cells correlated to the monkeys' judgements. The responses of a simple cell in the left V1 are shown in Fig. 2. The small receptive field of this cell was located to the bottom right of the fixation point (Fig. 2a). The cell responded to a vertical bar with directional selectivity (Fig. 2b), and it responded well for right eye stimulation (Fig. 2c), but not at all for left eye stimulation (Fig. 2d). The cell had no selectivity for stimulus disparity (Fig. 2e), but its activity was strongly modulated by the disparity of a masking pattern that was presented in conjunction with the stimulus bar.

An invisible patch, the colour of which was identical to the background, was placed on the receptive field. While a stimulus bar was crossing the invisible patch, the bar appeared to consist of two unconnected vertical segments. The cell did not respond to this stimulus pattern (Fig. 2f). It also did not respond when a visible grey patch was placed on the receptive field (Fig. 2g). However, the cell began to respond again when the patch had crossed disparity so that it appeared to be in front of the stimulus bar (Fig. 2h), but not when the patch had uncrossed disparity so that it appeared to be behind the stimulus bar (Fig. 2i). The cell also did not respond to a change in the disparity of the patch (Fig. 2j, k). These results indicate that the response properties of the simple cell are not 'simple' at all; the cell was obviously receiving inputs from other disparity-sensitive cells.

A total of 186 orientation-selective cells were tested, all of which

had receptive fields within 2° of the fixation point. Seventy-three cells were classified as simple cells and the remaining 113 as complex cells. Of these, 9 simple cells and 13 complex cells showed sufficient response properties to mediate amodal completion (Fig. 3a). The activity of these cells decreased considerably when the invisible patch was placed on their receptive fields (Fig. 3b), but was markedly restored when a visible grey patch was used with crossed disparity such that it appeared in front of the bar (Fig. 3c). However, the response was not restored when the patch had uncrossed disparity such that it appeared behind the stimulus bar (Fig. 3d, e). These differences cannot be attributed to differences in vergence angle, as there was no systematic difference in eye movement for the patch disparities. Out of these cells, six simple and eight complex cells showed no preference for the disparity of the stimulus bar, and two simple and three complex cells were classified

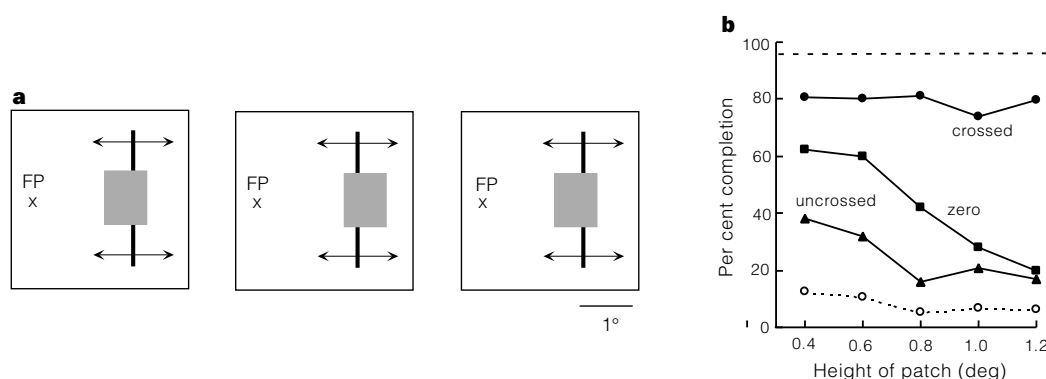


Figure 1 A stimulus pattern and behavioural responses. **a**, Diagrams of visual stimuli. For uncrossed viewing, the grey square patches in the left and middle patterns have uncrossed disparity, such that the patch appears to be in front of the segments. **b**, Monkeys' response for bars with patches of different binocular disparity. The

percentage of responses that are the same as those for a stimulus bar alone is plotted against the height of the patch. Responses for the bar alone (dashed line) and for two unconnected segments (open circle) are also shown for comparison.

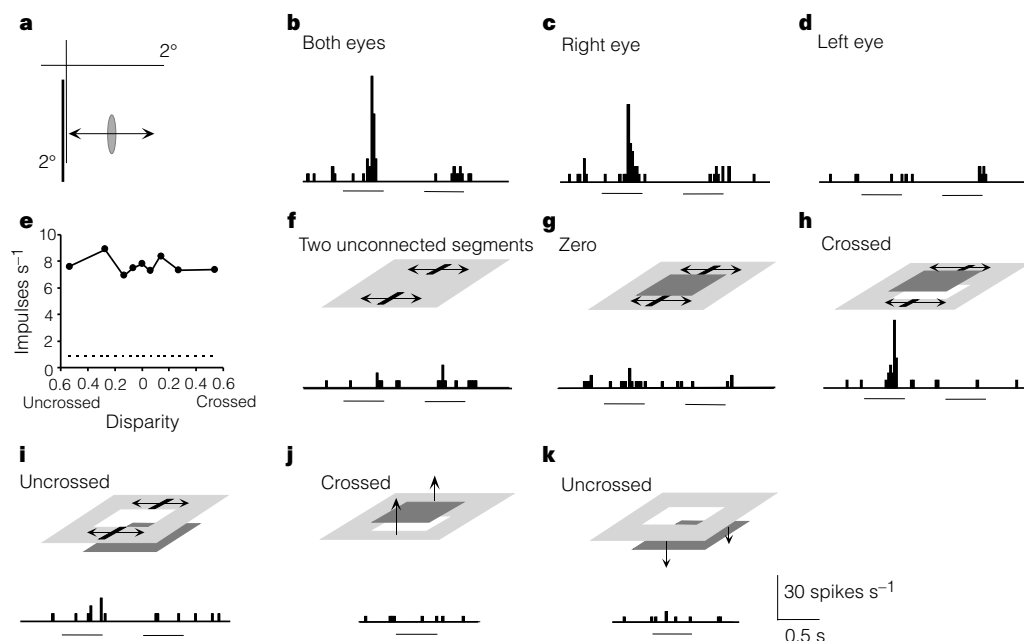


Figure 2 Responses of a simple cell. **a**, Ellipse represents the receptive field. **b–d**, Response for binocular stimulation, for right-eye stimulation and for left-eye stimulation, respectively. **e**, Disparity tuning curve. Dashed line represents spontaneous firing level. **f–i**, Responses for two unconnected segments, for a bar with a patch, for a bar behind a patch and for a bar in front of a patch, respectively. **j, k**, Response to the disparity change

of the patch. The left underlined sections below the peristimulus time histograms (**b–d**, **f–i**) represent the period during which the stimulus bar moved in the preferred direction, and the right underlined sections in the opposite direction. In **j** and **k**, the underlined sections represent the period during which the patch had crossed and uncrossed disparity, respectively.

as tuned excitatory cells. The remaining one simple and two complex cells were not fully tested for their selectivity. These cells might be able to represent contours or surfaces concealed behind other surfaces.

The concept of the classical receptive field in V1 has been challenged by many studies^{6–13}. The activity of V1 neurons evoked by an appropriate stimulus can be modulated by stimuli placed entirely outside the receptive field. This extra-receptive field contextual modulation has a latency in the range of ~80–100 ms, indicating that the modulation may be dependent upon feedback signals from extrastriate areas¹³. The response to the partially occluded bar represents a kind of contextual modulation, because two line segments outside the receptive field evoked the responses. However, the response latency for the bar behind the patch was not different from that for a stimulus bar alone. It is therefore likely that lateral connections in V1 underlie the response, or that the feedback signals come from areas very close to V1. In the real world, boundary segments often remain undetected. However, the visual system employs sophisticated mechanisms to complete such boundary segments. When an object is occluded by nearer objects, amodal contours are formed to aid object recognition^{1–5}. When the luminance contrast between an object and its background at some locations along the boundary is too low to be detected, modal or subjective contours are created to complete the segments^{3,4,14–17}. The cognitive approach has emphasized top-down processes to account for this completion^{15,16}. It might be thought that the amodal contours, in particular, might be based on a kind of cognitive

reasoning, as the contours are literally invisible. However, it has already been shown that neurons in V1 (ref. 18) and extrastriate visual area V2 (refs 19–21) respond to modal contours. Furthermore, the results of this study show that the amodal contours are also formed in V1. I conclude that border completion is carried out in very early stages of visual processing and that information available from the early stages is integrated for the higher-order interpretation of surface relations. □

Methods

Visual stimuli were presented on a CRT display (1,280 × 1,024 pixel resolution and a frame rate of 60 Hz). The screen was viewed at a distance of 60 cm. Stereo images were displayed side by side on the screen. A female (5.2 kg) and a male (4.7 kg) Japanese monkey viewed the screen through a hand-made prism haptoscope that allowed the horizontally displaced stereo images to be fused at a comfortable vergence angle¹³. Throughout the experiments, the disparity of a patch was produced by lengthening patches for the left and right eye in the appropriate horizontal directions, respectively. The movements of both eyes were recorded using infrared reflective oculography (see Supplementary Information). The spatial resolution was ~0.004° with a dynamic range of 4°. The monkeys were required to maintain fixation within a 0.2°-square fixation window and to keep the vergence angle within ±0.1°. A square fixation spot (0.05° × 0.05°) and other visual stimuli were presented only while fixation was maintained with a constant vergence angle.

Behavioural tests

In the training sessions, an invisible patch, the brightness of which was identical to that of the black background (0.0 cd m⁻²), was randomly presented at 2° to the right of the fixation point. The width of the patch was 0.74° and the height was randomly selected to be between 0.4° and 1.2° in steps of 0.2°. The image of the bar was separated into two line segments while the bar crossed the invisible patch. An extraneous grey patch (1.8 cd m⁻², 1.0° long and 0.74° wide) was always presented to prevent response to the patch during the test sessions. The binocular disparity of the extraneous patch was 0.14° uncrossed, zero or 0.14° crossed. The location of the extraneous patch, always outside the stimulus trajectory, was decided randomly. A trial began with the monkey pressing a lever. A stimulus bar (20.0 cd m⁻², 2° long and 0.05° wide) was presented at 1° to the right of the fixation point. 0.5 s later, the bar began to move to the right with a velocity of 2° per s for 1 s. The colour of the fixation spot was changed from red to yellow for 1 s. This was the choice cue. The monkey had to keep holding a lever for a full bar and release the lever for two unconnected line segments to obtain a drop of fruit juice. Test sessions were given after correct responses exceeded 90% for three consecutive days.

In the test sessions, 30% of trials were assigned as test trials and the remaining 70% as training trials. In the test trials, a grey patch (1.8 cd m⁻²) was placed on the trajectory and the extraneous patch was not presented. The grey patch had 0.14° of uncrossed disparity (to the back), zero disparity (at the same depth) or 0.14° of crossed disparity (in front). The disparity and the height of the patch were randomly selected from trial to trial. If the monkey held the lever, the partially occluded bar was regarded as being perceptually completed. All the responses were rewarded in the test trials and a total of 50 responses were obtained for each condition.

Unit recording

Single cells were recorded with glass-coated elgiloy electrodes (2.5–3.5 M at 1 kHz) during a passive fixation task. Selectivity for orientation and line length was initially examined for each isolated cell. End-stopped cells and cells with no orientation selectivity were not tested further. By moving the stimulus bar, the location and extent of the receptive field was estimated for excitatory responses. The estimated receptive field dimensions were confirmed by flashing stimulus bars inside and outside this area.

The same stimulus pattern as in the behavioural tests was employed. The luminance of the bar, the visible grey patch and the background was 20, 1.8 and 0.0 cd m⁻², respectively. The size of the smallest patch was 1.0° × 0.74°. If an isolated cell responded to two unconnected line segments (a bar with an invisible patch), the size of the patch was enlarged in steps of 0.19° × 0.14° until the cell did not respond to the segments. The velocity of the bar was 2° per s. However, it was 5° per s when testing the preference for the disparity of the bar, so that the bar moved across each eye's receptive field.

The average firing rate during the 500-ms stimulus period was calculated on the basis of at least seven trials and the average spontaneous firing rate immediately before stimulation was subtracted. For statistical analysis, an *F*-test was employed and a significant criterion level of *P* < 0.05 was used.

All the procedures were carried out in accordance with NIH guidelines and were approved by the Animal Care and Use Committee of the National Institute of Bioscience and Human Technology, Japan.

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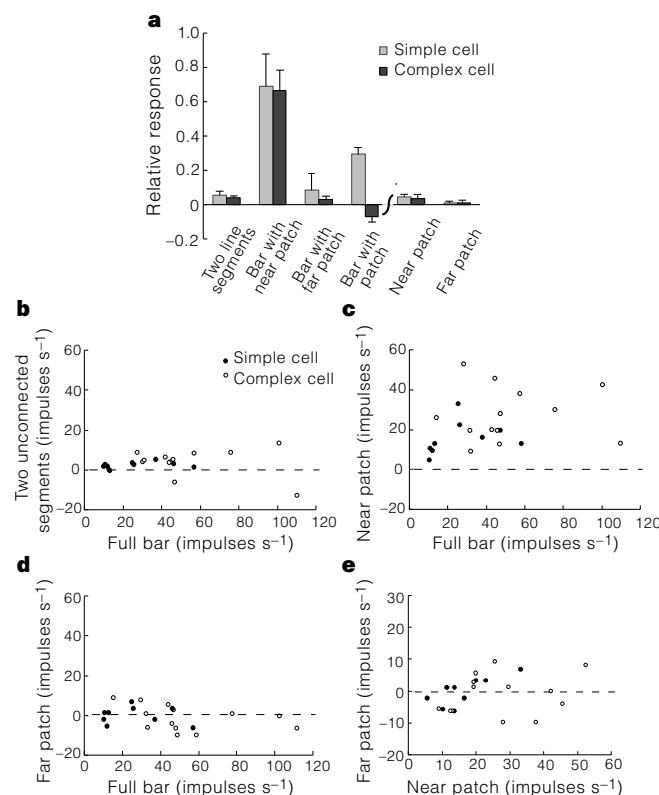


Figure 3 Comparison of response strength. **a**, Variation in responses among single units for bar with different disparity. Responses are normalized to those for the bar alone. The responses for the disparity change of the patch from zero to crossed and from zero to uncrossed are also shown for comparison. **b**, Responses to two unconnected line segments plotted against the response to the full bar alone. **c**, Responses to bar with a patch with crossed disparity plotted against the response to the full bar alone. **d**, Response to bar with a patch with uncrossed disparity plotted against the response to the full bar alone. **e**, Response to bar with a patch with uncrossed disparity plotted against the response to the bar with a patch with crossed disparity.

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Expression of the transcription factor Δ FosB in the brain controls sensitivity to cocaine

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Acute exposure to cocaine transiently induces several Fos family transcription factors in the nucleus accumbens¹, a region of the brain that is important for addiction^{2,3}. In contrast, chronic exposure to cocaine does not induce these proteins, but instead causes the persistent expression of highly stable isoforms of Δ FosB^{4–6}. Δ FosB is also induced in the nucleus accumbens by repeated exposure to other drugs of abuse, including amphetamine, morphine, nicotine and phencyclidine^{7–10}. The sustained accumulation of Δ FosB in the nucleus accumbens indicates that this transcription factor may mediate some of the persistent neural and behavioural plasticity that accompanies chronic drug

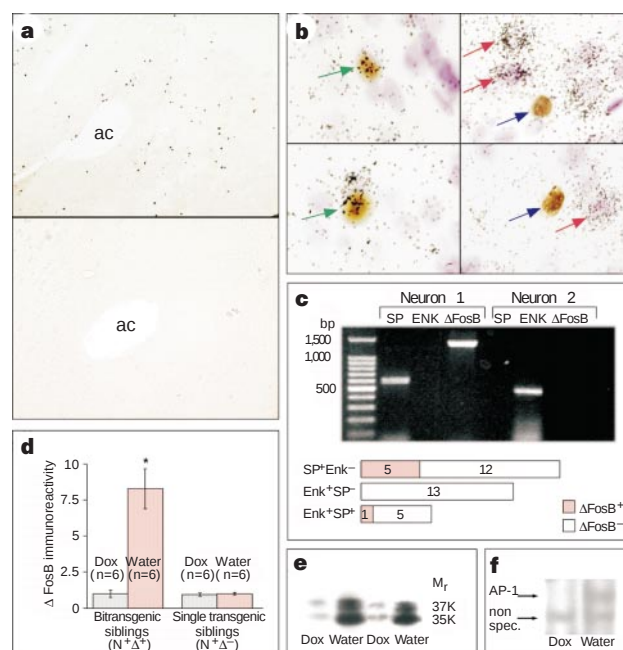
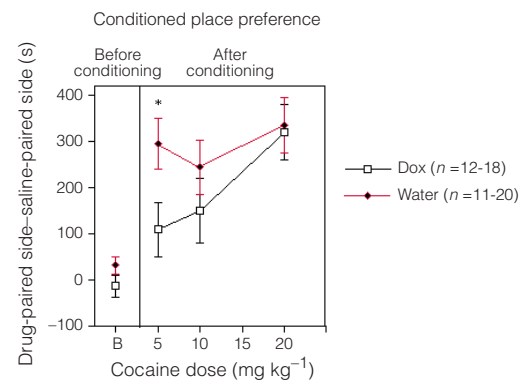


Figure 1 Δ FosB expression in NAc of bitransgenic NSE-tTA $\times$ TetOp- Δ fosB ($N^+\Delta^+$) mice. **a**, Immunohistochemistry showing Δ FosB induction in bitransgenic but not single transgenic NSE-tTA ($N^+\Delta^-$) mice (original magnification 200 $\times$). ac, anterior commissure. **b**, Colocalization of Δ FosB with dynorphin, but not enkephalin, in $N^+\Delta^+$ mice. Left: green arrows, Δ FosB-positive, dynorphin-positive NAc neurons. Right: red arrows, enkephalin-positive, Δ FosB-negative NAc neurons; blue arrows, Δ FosB-positive, enkephalin-negative NAc neurons. **c**, Single-cell RT-PCR confirming Δ FosB expression exclusively in substance-P-positive neurons. Top, representative gel. Bottom, number of neurons double-positive for neuropeptide and Δ FosB. **d**, Bar graph and western blot (**e**) showing Δ FosB induction in $N^+\Delta^+$, but not $N^+\Delta^-$, mice switched to water (asterisk: $t = 5.27$, $P < 0.0005$, Student's t -test). **f**, Gel shift showing induction of AP-1 binding activity in Δ FosB-expressing mice. non spec., non-specific band. Dox, doxycycline.

exposure¹. Using transgenic mice in which Δ FosB can be induced in adults in the subset of nucleus accumbens neurons in which cocaine induces the protein, we show that Δ FosB expression increases the responsiveness of an animal to the rewarding and locomotor-activating effects of cocaine. These effects of Δ FosB appear to be mediated partly by induction of the AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole) glutamate receptor subunit GluR2 in the nucleus accumbens. These results support a model in which Δ FosB, by altering gene expression, enhances sensitivity to cocaine and may thereby contribute to cocaine addiction.

We used the tetracycline gene-regulation system¹¹ to develop a mutant mouse that inducibly overexpresses Δ FosB¹². In the absence of the tetracycline derivative doxycycline, the tetracycline transactivator tTA (encoded by one transgene) binds to the tetracycline responsive promoter (TetOp) and thereby activates transcription of Δ fosB (encoded by a second transgene) in bitransgenic animals. We used the neuron-specific enolase (NSE) promoter to restrict tTA (and hence Δ FosB) expression to the nervous system¹². One line of NSE-tTA mice (line A), when crossed with TetOp- Δ fosB mice, showed robust and highly selective expression of Δ FosB throughout the striatum (including the core and shell of the nucleus accumbens (NAc), Fig. 1a), with very low expression elsewhere in brain or peripheral tissues¹². The targeting of the striatum is presumably due to the insertion site of the NSE-tTA gene in this line. Moreover, only one subset of NAc medium spiny neuron is targeted in these animals. Two independent methods—dual-labelling immunohistochemistry-*in situ* hybridization (Fig. 1b) and single-cell polymerase chain reaction with reverse transcription (RT-PCR; Fig. 1c)—



treatment¹⁹; third, glutamate receptor antagonists block the induction of certain behavioural adaptations to repeated drug exposure^{14,16}; and finally, the genes for specific glutamate receptor subunits, including GluR2, contain AP-1 sites^{20–23}. Expression of Δ FosB in bitransgenic mice significantly increased levels of GluR2 (an AMPA receptor subunit) by more than 50% in the NAc (Fig. 4a). Removal of doxycycline from single transgenic mice did not cause this effect. Δ FosB had no effect on levels of several other glutamate receptor subunits (Fig. 4b). Moreover, GluR2 induction was not observed in dorsal striatum ($93 \pm 19\%$ of dox animals, $n = 6$) or other brain regions (not shown). Induction of GluR2 in NAc of Δ FosB-expressing mice could represent a direct effect of Δ FosB on the GluR2 gene, as a portion of the GluR2 gene promoter that contains a consensus AP-1 site²³ binds Δ FosB (Fig. 4c).

Unlike other AMPA receptor subunits, GluR2 undergoes RNA editing, which alters a genetically coded glutamine into an arginine located in the pore region of the molecule^{24,25}. The Q $\rightarrow$ R change makes AMPA receptors that contain the GluR2 subunit virtually impermeable to calcium ions and decreases their overall current. Therefore, a selective increase in GluR2 in the NAc could have important functional consequences. To determine whether the Δ FosB-induced increase in GluR2 levels could account for the enhanced behavioural sensitivity to cocaine in the Δ FosB-expressing mice, we microinjected a recombinant herpes simplex virus vector encoding GluR2 (HSV-*gluR2*) directly into the NAc of rats and tested cocaine's rewarding properties using place conditioning. For comparison, we injected vehicle or viral vectors encoding GluR1 (HSV-*gluR1*), LacZ (HSV-*lacZ*, which encodes β -galactosidase, a control protein) or the unedited version of GluR2

(HSV-*gluR2(Q)*); GluR2Q retains permeability to calcium ions and consequently resembles GluR1 electrophysiologically. With bilateral injections of vehicle or HSV-*lacZ* into the NAc, a threshold dose of cocaine (1.25 mg kg^{-1}) failed to establish a place preference (Fig. 5a). In contrast, HSV-*gluR2*-mediated overexpression of GluR2 in the NAc markedly enhanced the rewarding effects of this low dose of cocaine. Overexpression of GluR1 had the opposite effect: rats injected with HSV-*gluR1* spent significantly less time in the drug-paired environment (that is, cocaine was aversive). Overexpression of GluR2(Q) also tended to make cocaine aversive. These HSV vectors mediate transgene overexpression in a moderate number of neurons in a highly localized area of about 1 mm in diameter around the injection site^{17,26}. This is shown for GluR2 and LacZ in Fig. 5b and c, respectively. Moreover, these HSV treatments are not associated with any detectable toxicity^{17,26} (for example, no gliosis, based on cresyl violet staining (Fig. 5d)).

Our data show that selective induction of Δ FosB in dynorphin/substance-P-containing neurons of the NAc and dorsal striatum in the adult is sufficient to increase an animal's responsiveness to the rewarding and locomotor-activating effects of cocaine. This finding is consistent with the observation that cocaine is also more reinforcing in 5HT_{1B}-receptor knockout mice, which show a compensatory increase in Δ FosB in the NAc²⁷. Induction of Δ FosB by chronic cocaine exposure could, therefore, be a mechanism for relatively long-lived increases in sensitivity to the stimulant and rewarding effects of the drug. Such increases in sensitivity, which accompany repeated drug exposure, may contribute to human cocaine addiction^{14–16}. FosB knockout mice are also more sensitive to some of the initial behavioural effects of cocaine (which may seem paradoxical given the results of this study), although the knockouts fail to sensitize to repeated cocaine exposures⁵. However, the interpretability of the knockout is limited in several ways. First, these mice lack not only Δ FosB but also full-length FosB, which is induced acutely by cocaine⁴. Second, as these are constitutive knockout mice,

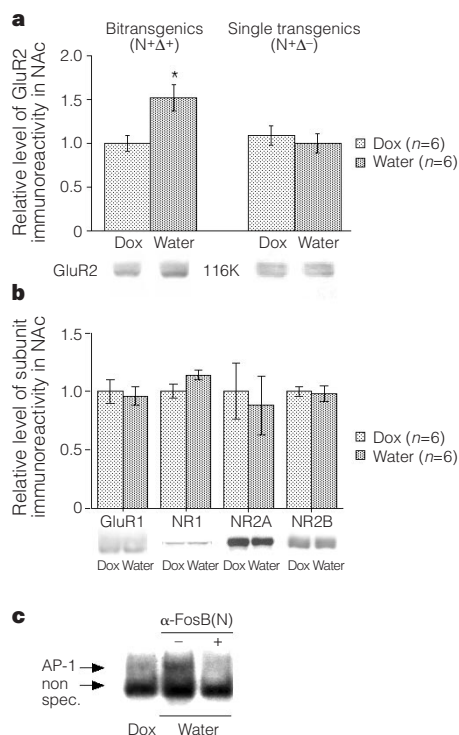


Figure 4 Effect of Δ FosB expression on levels of glutamate receptor subunits and AP-1 binding in NAc. **a**, **b**, Levels of glutamate receptor subunits in NAc, measured by western blot, relative to dox group. **a**, Increased GluR2 levels in bitransgenics (asterisk, $t = 2.82$, $P < 0.05$, Student's t -test), but not single transgenics, upon switching to water (that is, upon Δ FosB induction). **b**, No effect of Δ FosB on other glutamate receptor subunits. NR, NMDA receptor subunit. **c**, Gel shift of NAc extract showing increased binding to a GluR2 gene promoter fragment, which contains an AP-1 site, upon Δ FosB expression, and its disruption by an anti- Δ FosB(N-terminal) antibody (α -FosB(N)).

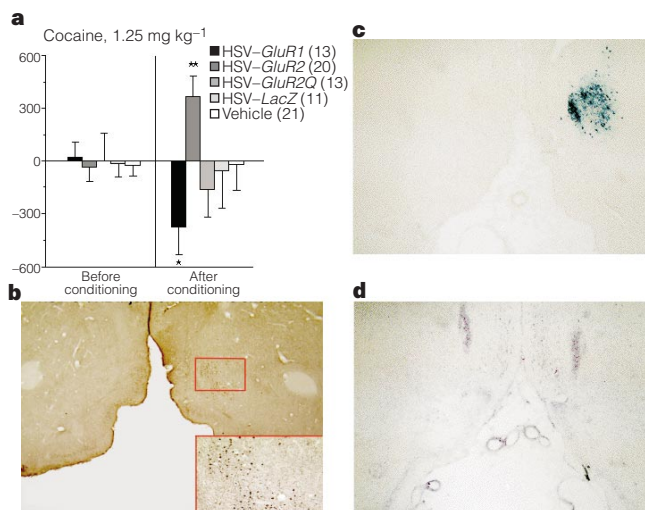


Figure 5 Effect of HSV vectors in NAc on place conditioning to cocaine. **a**, Effect of a threshold cocaine dose (1.25 mg kg^{-1} , i.p.) depended on vector treatment (treatment $\times$ sessions interaction: $F_{4,77} = 3.04$, $P < 0.05$). Cocaine had no effect in rats injected with vehicle or HSV-*lacZ*. Rats given HSV-*gluR2* injections spent more time in the cocaine-paired chamber, whereas rats given HSV-*gluR1* spent less time in the cocaine-paired chamber. Asterisk, $P < 0.05$; double asterisk, $P < 0.01$; Fisher's t -test. **b**, Immunohistochemistry showing GluR2 induction three days after unilateral HSV-*gluR2* injection into NAc (original magnification 25 $\times$, inset 200 $\times$). ac, anterior commissure. **c**, Expression of β -galactosidase three days after unilateral HSV-*lacZ* injection into NAc (25 $\times$). **d**, Adjacent cresyl-violet-stained section from **c** showing lack of gliosis after viral treatment (25 $\times$).

the loss of Δ FosB and FosB occurs from the earliest stages of development and in all tissues. Third, the knockouts show enhanced sensitivity to initial cocaine exposure; this is difficult to relate to Δ FosB, which is induced only in response to chronic cocaine exposure. These results underscore the caution that must be used in interpreting data from constitutive mutant mice and highlight the advantages of inducible, tissue-specific mutants like those used here.

We also show, by viral-mediated gene transfer, that increased GluR2 expression in the NAc can account for the enhanced sensitivity to cocaine's rewarding effects seen in the Δ FosB-expressing mice. While GluR2 is probably just one of many targets regulated by Δ FosB in the NAc, our results support a model in which chronic cocaine exposure results in the gradual accumulation of Δ FosB in this brain region, which then causes increased expression of GluR2. An important goal for future research is to understand how increased GluR2 expression in the NAc enhances reward mechanisms. The increase in GluR2 could account for the reduced sensitivity of NAc neurons to AMPA seen after chronic cocaine exposure, as well as for reductions in Ca^{2+} flux seen in these neurons¹⁹. Moreover, inhibition of NAc neurons has been related directly to drug reward²⁸. Together, the results indicate that Δ FosB may mediate relatively long-lived changes in gene expression that increase sensitivity to cocaine and thereby contribute to the development of cocaine addiction. □

Methods

Mice

Male mice derived from NSE-tTA line A and TetOp- Δ foxB line 11 (ref. 12) were maintained on an outbred background (50% ICR, 50% 57B16 $\times$ SJL). Unless otherwise indicated, all mice were conceived and raised on 200 $\mu\text{g ml}^{-1}$ doxycycline (Sigma) to suppress Δ FosB expression during development. Littermates were divided at weaning: half remained on doxycycline and half were switched to water, and the animals were used 11 weeks later. All data are expressed as mean $\pm$ s.e.m.

Locomotor activity

Locomotor activity (Fig. 2a) was studied as described⁵ except that mice were habituated for 4 days before receiving cocaine. As before, activity was measured for 10 min to avoid stereotypy⁵.

Place conditioning

An unbiased conditioning protocol⁵ was used, with the following modifications. On day 1, mice explored the three chambers freely for 20 min; they did not show a preference for any chamber before conditioning. During the next three days mice were confined to one large chamber for 20 min, where they received saline (1 ml kg^{-1} , i.p.). Four hours later, they were confined to the other side for 20 min, where they received cocaine. On day 5, mice were placed in the central chamber and allowed to move freely in all three chambers for 20 min. The time they spent in the cocaine-paired chamber minus the time in the saline-paired chamber provided a measure of place conditioning. Place conditioning of rats was as described^{17,26}.

HSV viral surgery

Complementary DNAs for *gluR1*, *gluR2*, *GluR2(Q)* and *lacZ* were inserted into the HSV amplicon HSVPrpUC and packaged into virus with the helper 5d11.2 (ref. 17). Transgene expression was regulated by the constitutive promoter for the HSV immediate-early gene *IE4/5*. HSV vectors were injected into the medial NAc as described¹⁷.

Western blotting and gel shifts

NAc and dorsal striatum were excised from 1-mm-thick coronal slices of mouse brain using 15- and 14-gauge syringe needles, respectively. Western blotting was performed as described⁵ with the following primary antibodies, the specificity of which has been established previously: rabbit anti-Fra (Fos-related antigen) (1:4000), rabbit anti-GluR1 (1:1000, Chemicon AB 1504), mouse anti-GluR2/4 (1:3333, Pharmingen 60011A), mouse anti-NR1 (1:2000, Pharmingen 60021A), rabbit anti-NR2A (1:1000, Chemicon AB 1555P) and rabbit anti-NR2B (1:1000, Chemicon AB 1557P). Equal loading and transfer of proteins was confirmed by the following: (1) all blots were stained with amido black; (2) levels of actin ($92 \pm 21\%$ of dox animals, $n = 6$) and of neurofilament-160 ($99 \pm 14\%$, $n = 6$) were not altered by Δ FosB expression; and (3) blots used to measure GluR2 (which was increased by Δ FosB) were also used to measure levels of NMDA receptor subunits (which were not affected by Δ FosB). Gel shifts were performed as described⁴ with 20 μg of total protein and a ^{32}P -labelled AP-1 oligonucleotide. Gel shifts shown in Fig. 4c were performed in an identical manner, except that a 19mer (GACCCAGTGACTAAGGCAA)

containing an AP-1 site (underlined) located $\sim 1.2\text{-kb}$ 5' of the transcription initiation region in the GluR2 gene was used²⁵. The specificity of the AP-1 bands seen with both probes was established by cold competition and supershift studies⁴⁻⁶.

Histology

Immunohistochemical analysis of Δ FosB and GluR2 was done as described^{5,26}. Coronal sections (40 μm) were labelled with a rabbit polyclonal anti-FosB(amino-terminal) antibody (1:5000, Pharmingen 6011A), and then processed with standard techniques. β -Galactosidase and cresyl violet staining of HSV-injected sections was done as described^{17,26}.

Dual-label immunohistochemistry-in situ hybridization

Δ FosB expression was localized to a subset of NAc neuron using a dual-labelling procedure. 20- μm free-floating coronal sections were stained for the Δ FosB transgene (as described above). After mounting on slides, sections were prepared for *in situ* hybridization⁷ using ^{35}S -labelled riboprobes for enkephalin (a 476-bp probe corresponding to nucleotides 314–790 (ref. 29)) or dynorphin (a 1714-bp probe corresponding to nucleotides 272–1986) (ref. 30). Following film autoradiography, tissue sections were dipped in emulsion, exposed for 2–5 weeks and counterstained with cresyl violet.

Single-cell RT-PCR

Striatal (including NAc) neurons from ~ 6 -week-old mice were acutely dissociated, and single-cell RT-PCR was performed²⁹ to detect neuropeptide or Δ FosB transgene messenger RNA. The primer sequences used in these reactions have been published elsewhere^{12,29}.

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Function of Rieger syndrome gene in left–right asymmetry and craniofacial development

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Rieger syndrome, an autosomal dominant disorder, includes ocular, craniofacial and umbilical abnormalities. The *pitx2* homeobox gene, which is mutated in Rieger syndrome^{1,2}, has been proposed to be the effector molecule interpreting left–right axial information from the early embryonic trunk to each organ^{3–7}. Here we have used gene targeting in mice to generate a loss-of-function allele that would be predicted to result in organ randomization or isomerization. Although *pitx2*^{−/−} embryos had abnormal cardiac morphogenesis, mutant hearts looped in the normal direction. *Pitx2*^{−/−} embryos had correctly oriented, but arrested, embryonic rotation and right pulmonary isomerism. They also had defective development of the mandibular and maxillary facial prominences, regression of the stomodeum and arrested tooth development. *Fgf8* expression was absent, and *Bmp4* expression was expanded in the branchial-arch ectoderm. These data reveal a critical role for *pitx2* in left–right asymmetry but indicate that *pitx2* may function at an intermediate step in cardiac morphogenesis and embryonic rotation.

Although Rieger syndrome results from haploinsufficiency⁸, the *pitx2*^{hd} allele, which deleted exon 5 and part of exon 6, including the homeobox (Fig. 1a–c), did not result in an obvious haploinsufficient phenotype. Analysis of 239 embryos from F1 intercrosses revealed a loss of *pitx2*^{hd} mutants at embryonic day (E) 14.5, probably from haemodynamic compromise (see below). To investigate left–right axis determination, we focused on cardiac looping, embryonic rotation and individual organ situs^{9,10}.

At E8.5, *pitx2*^{hd/−} hearts had looped rightwards and expression of

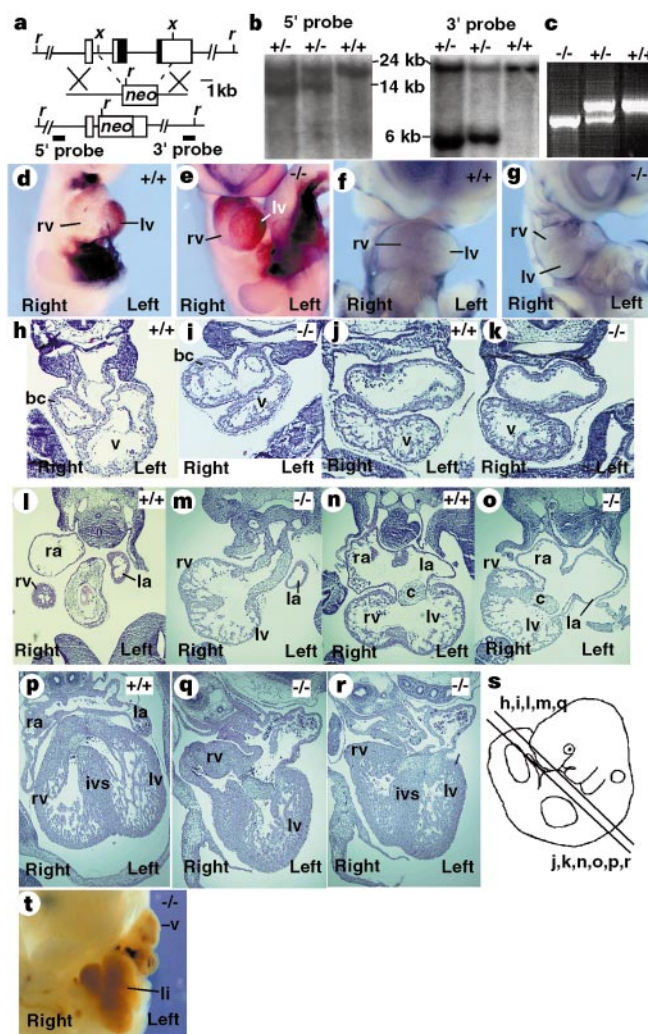


Figure 1 Targeting strategy and cardiac phenotype. **a**, Partial *pitx2* structure. Homeobox, black. Targeting vector (middle), *pitx2*^{hd} (bottom). r, *EcoRV*; x, *XhoI*. **b**, Southern blots. **c**, PCR genotyping. **d–g**, *eHAND* (**d**, **e**), *Bmp4* (**f**, **g**) whole-mount *in situ* hybridization. **h–r**, Rostral (**h**, **i**, **l**, **m**, **q**) and caudal (**j**, **k**, **n**, **o**, **p**, **r**) transverse sections: E9.5 (**h–k**), E10.5 (**l–o**), E12.5 (**p–r**). **s**, Planes of section for **h–r**. **t**, E12.5 embryo. bc, bulbus cordis; c, cushions; ivs, interventricular septum; la, lv left atrium, ventricle; li, liver; ra, right atrium; rv, right ventricle; v, ventricle.

eHAND, a left ventricle marker¹¹, was maintained (Fig. 1d, e). Although early stages of asymmetric cardiac morphogenesis were spared in *pitx2*^{hd/−} embryos, at E10.5, mutant ventricles were displaced rightwards (Fig. 1f, g). Sections of mutants showed rightwards deviation of the bulbus cordis at E9.5 (Fig. 1h, i, s) and a normal atrioventricular relationship (Fig. 1j, k, s). At E10.5, ventricular deviation was intensified, with rightward, dorsal distortion of the atrioventricular canal and outflow tract (Fig. 1l–o, s). Mutant ventricles formed on the same dorsal–ventral plane as the outflow tract (Fig. 1l, m). Despite these abnormalities, which may represent a delay in cardiac looping¹¹, *pitx2*^{hd/−} embryos formed distinct ventricles (Fig. 1p–s). At E12.5, *pitx2*^{hd/−} hearts developed ectopically in a ventral, left-sided location with the apex directed cranial and rightwards (Fig. 1t), perhaps secondarily to defective embryonic rotation (see below; Fig. 2f, g). Thus, *pitx2* acts after the ventricular region of the heart tube bends to the right.

Pitx2^{hd/−} embryos correctly initiated counterclockwise rotation; however, turning was arrested, suspending the mutant lower trunk

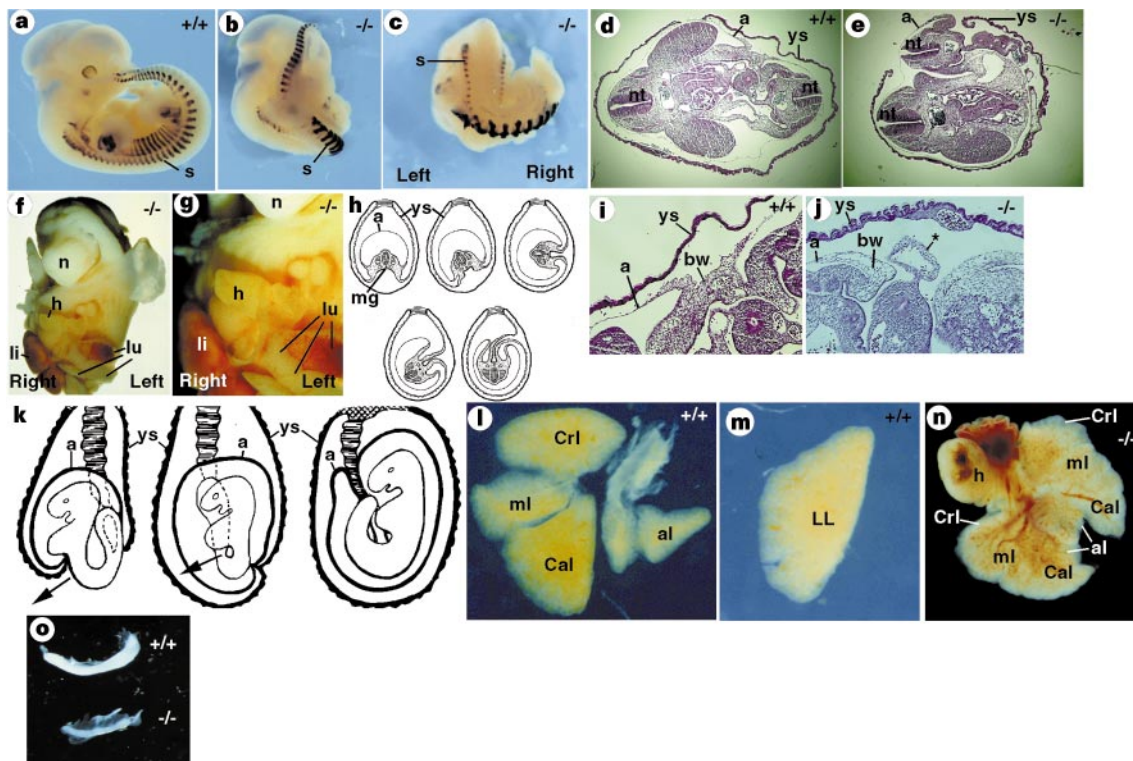


Figure 2 Rotation and pulmonary phenotypes. **a–c**, Lateral (**a**, **b**) and posterior (**c**) views of embryos probed with myogenin. **d**, **e**, Transverse sections. **f**, **g**, Low- (**f**) and high-power (**g**) views of E14.5 embryo. **h**, Rotation and extra-embryonic membranes. **i**, **j**, Transverse sections. Asterisks mark midgut. **k**, Defective turning and evisceration (arrow).

l, **m**, Lungs in an E14.5 embryo. **n**, Lungs and heart of an E12.5 embryo. **o**, E14.5 spleens. a, amnion; al, accessory lobe; bw, body wall; CrL, cranial lobe; Cal, caudal lobe; h, heart; li, liver; lu, lung; mg, midgut; ml, middle lobe; n, nasal; nt, neural tube; s, somites; st, stomach; ys, yolk sac.

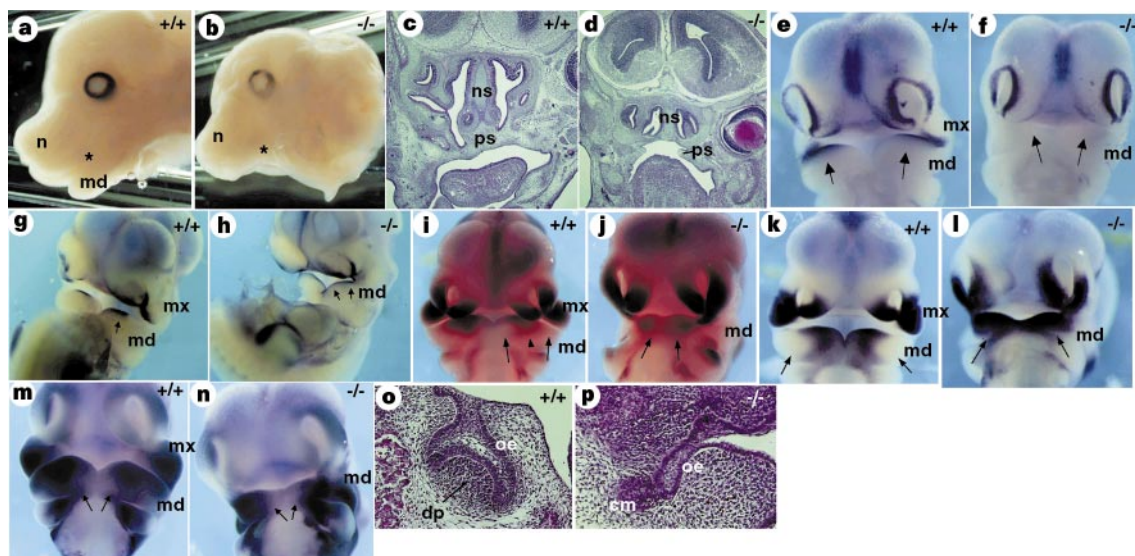


Figure 3 Craniofacial phenotype. **a**, **b**, E14.5 heads, missing stomodeum (asterisk). **c**, **d**, E14.5 coronal sections. **e–n**, E10.5 whole-mount *in situ* hybridization. **e**, **f**, Absent *Fgf8* expression (arrows). **g**, **h**, Expanded *Bmp4* expression (arrows). **i**, *Msx1* expression in mandibular process (md) (arrowhead); excluded areas (arrows). **j**, The mutant embryo shows diffuse *msx1* expression (arrows). **k**, **l**, *Msx2* expression in md is restricted

(arrows). Expanded *msx2* expression in mutant md (arrows). **m**, **n**, *Prx-1* whole-mount. **o**, **p**, E14.5 transverse sections. cm, condensed mesenchyme; dp, dental papilla; mx, maxillary process; n, nasal structures; ns, nasal septum; oe, oral epithelium; ps, palatal shelf.

to the right of the upper trunk (Fig. 2a–e). Expressivity was variable, as 10% of mutants had delayed turning. Therefore, *pitx2* acts after the direction of turning (or heart looping) has been determined. Consistent with their multistep nature¹², cardiac mor-

phogenesis and embryonic rotation appear to be regulated at multiple levels.

The lungs develop posterior to the heart and lateral to the midline. In *pitx2*^{hd/-} embryos, the lungs and abdominal contents

formed ventrally, inferior to the heart and to the left of the body axis (Fig. 2f, g). The turning embryo is enveloped in extra-embryonic membranes¹² (Fig. 2h). Sections through E10.5 mutant embryos in which rotation was minimally affected showed a patent umbilical ring and evisceration of abdominal contents (Fig. 2i, j), indicating that delayed rotation may result in organ evisceration (Fig. 2k). A defect in the ventral body wall, which expresses *pitx2* (refs 1, 3), may also contribute to evisceration.

The left lung has one lobe and the right lung has four lobes (Fig. 2l, m). *Pitx2*^{hd/-} mice show right pulmonary isomerism (Fig. 2n), whereas the stomach maintains its left-sided position and has normal morphology. *Pitx2*^{hd} mutants have splenic hypoplasia, also reported in patients with laterality defects^{13,14} and in ActRIIB mutant mice¹⁵ (Fig. 2o).

In the craniofacial region, the mutant stomodeum regressed by E14.5 (Fig. 3a, b). *Pitx2*^{hd/-} embryos had abnormal development of the maxillary and mandibular facial prominences and cleft palate (Fig. 3c, d). A marker of the branchial-arch ectoderm, *fgf8*, which is expressed broadly at E10.0¹⁶, was absent in this ectoderm of *pitx2*^{hd} mutants (Fig. 3e, f). *Bmp4* is expressed in medial oral ectoderm at E10.0; at E10.5, expression extends laterally to overly non-odontogenic mesenchyme¹⁶. In mutants, *Bmp4* expression expanded laterally into oral ectoderm, where it is not normally expressed (Fig. 3g, h). Expression of *msx1* and *msx2*, which are targets of *Bmp* signalling pathways¹⁷, was also expanded in mutants (Fig. 3i–l), whereas *prx-1* maintained its normal expression (Fig. 3m, n). Previous studies have shown that *fgf8* signalling induces and *Bmp* signalling inhibits dental mesenchyme specification¹⁶. *Pitx2*^{hd/-} mandibular teeth arrested as tooth buds, and maxillary teeth arrested at the placode stage (Fig. 3o, p).

Pitx2 is expressed in E10.5 periocular mesenchyme¹ (Fig. 4a), and at E15.5 in mesenchyme surrounding the retina, presumptive cornea, eyelids and extraocular muscles (Fig. 4b, c), indicating a requirement for *pitx2* in morphogenesis or specification of cranial neural-crest-derived periocular mesenchyme¹⁸. Examination of whole eyes and sections (Fig. 4d–i) revealed that the eyes of *pitx2*^{hd/-} mice had a displaced, irregular pupil (Fig. 4d, e) and lacked an anterior chamber, differentiated cornea and extraocular muscles (Fig. 4f–i). Instead of a thin, flattened cornea, *pitx2*^{hd} mutants had undifferentiated mesenchymal cells that expressed *Imx1b*, a marker of periocular mesenchyme (Fig. 4j, k). Mutants lacked a corneal endothelium, as shown by histology and N-

cadherin staining (Fig. 4h, i, l, m and not shown), and this is likely to be a cause of the anterior chamber defects. Thus, *pitx2* is essential for proper differentiation of periocular mesenchyme, indicating that this function of *pitx2* underlies Axenfeld–Rieger ocular pathologies.

Loss of *pitx2* function results in right pulmonary isomerization, supporting previous proposals that *pitx2* provides leftness to the pulmonary primordia¹⁹. In contrast, early aspects of cardiac looping and embryonic rotation are unaffected, whereas later events are disrupted, indicating that there may be an intermediate step between axial signalling and *pitx2* function. In oral ectoderm, *pitx2* positively regulates *fgf8* and restricts *Bmp4*, indicating that *pitx2* may regulate local cell signalling during asymmetric morphogenesis. □

Methods

Gene targeting in embryonic stem cells.

Phage clones from a 129/Sv genomic library were mapped. The targeting vector replaced a *XhoI* fragment with *neomycin* and introduced a novel *EcoRV*. Embryonic stem-cell culture and generation of chimaeras were performed as described²⁰.

Embryo analysis.

Whole-mount *in situ* hybridization, sectioning and staining of tissue sections were performed as described²⁰.

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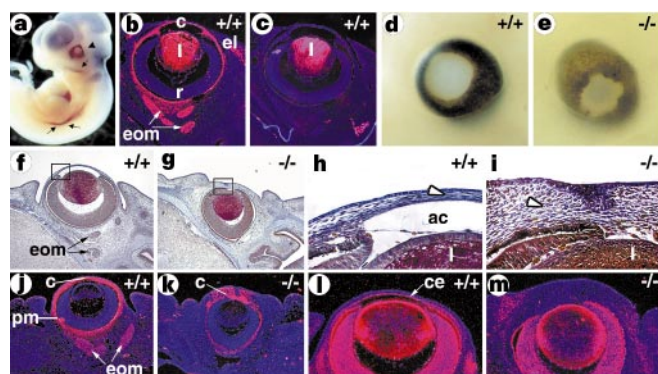


Figure 4 Ocular phenotype. **a, b**, E10.5 (**a**) and E15.5 (**b**) *pitx2* expression *in situ*: periocular mesenchyme (arrowhead), oral ectoderm (arrows), body wall (arrows), corneal stroma (c), eyelid mesenchyme (el), extraocular muscles (eom). Retina (r) and lens (l) are negative. **c**, *Pitx2* sense probe. **d, e**, Whole-mount eyes. **f, g**, Mallory's Trichrome-stained transverse sections. **h, i**, 40× magnification of boxed areas in **f** and **g**. Flattened corneal stroma (arrowhead, **h**), anterior chamber (ac), stellate presumptive corneal stromal cells (arrowhead, **i**). **j–m**, E14.5 *Imx1b* (**j, k**) and *n-cadherin* (**l, m**) *in situ* hybridization. Corneal endothelium, ce.

Pitx2 regulates lung asymmetry, cardiac positioning and pituitary and tooth morphogenesis

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Pitx1 (refs 1–3) and *Pitx2* (refs 4, 5) are highly homologous, bicoid-related transcription factors. *Pitx2* was initially identified as the gene responsible for the human Rieger syndrome⁴, an autosomal dominant condition that causes developmental abnormalities. *Pitx2* is asymmetrically expressed in the left lateral-plate mesoderm^{5–11}, and mutant mice with laterality defects show altered patterns of *Pitx2* expression that correlate with changes in the visceral symmetry (*situs*). Ectopic expression of *Pitx2* in the right lateral-plate mesoderm alters looping of the heart and gut and reverses body rotation in chick and *Xenopus* embryos^{6–11}. Here we describe the phenotype of *Pitx2* gene-deleted mice, characterized by defective body-wall closure, right pulmonary isomerism, altered cardiac position, arrest in turning and, subsequently, a block in the determination and proliferation events of anterior pituitary gland and tooth organogenesis. Thus, *Pitx2* is a transcription factor that encodes 'leftness' of the lung.

On the basis of potential biological roles for *Pitx2* (refs 5–13) (Fig. 1a), we devised a gene-deletion strategy by replacing the entire

Pitx2 homeobox and carboxy-terminal coding information with an in-frame *LacZ* sequence (Fig. 1b). Two homologous recombinant embryonic stem-cell lines were used to generate chimaeric mice from which *Pitx2*^{−/−} mice were ultimately obtained (Fig. 1c). Analysis confirmed that expression of the *LacZ* transcript accurately reflects the pattern of *Pitx2* expression in the gene-deleted mouse (Fig. 1d). The *Pitx2*^{−/−} mouse exhibits a severe phenotype, with initial embryonic lethality (~35%) on embryonic day (E) 9.25–10.25 and continual attrition of mice with survival up to E15. There is a failure to close the ventral body wall and thorax, apparent by E9.5 and independent of gut herniation; by E12 both thoracic and abdominal organs are positioned externally (Fig. 1e, f). The defect appears to reflect a primary defect of ventral body-wall closure; although ~2% of *Pitx2*^{+/−} newborn mice exhibit abnormalities, many display thinning of the ventral body wall in the absence of visible visceral herniation.

Our data is most consistent with an initially proper orientation of turning and a subsequent arrest in turning of the posterior part of the tail by E9–E10 in about 50% of the examined mutants (Fig. 2a). In embryos surviving to E13.5, there is an additional defect, independent of the direction of turning; maintenance of posterior lordosis occurs in ~70% of mutants, with variable severity, linked to the ventral body-wall defect (Figs 1e, 2b). The noticeable bending of the upper body axis in all mutant embryos (Fig. 2c) may also reflect an arrest in the turning process. Furthermore at E12, the stomach often turns to the left, rather than the right. Expression of *nodal* and *lefty-1* (refs 2–6), components of the left-specific signal cascade, is uninfluenced by deletion of the *Pitx2* gene.

Development of the cardiovascular system is altered. While there is no evidence of abnormalities in cardiac development, ~50% of E10 mutants exhibit positioning on the right rather than the midline (Fig. 2d, e). Expression of *eHAND*, a specific marker for the systemic ventricle^{14,15}, was not altered in *Pitx2*^{−/−} mice and there is no evidence for altered direction of looping of the cardiac tube, as the systemic ventricle remains positioned on the left side of the right-positioned heart (Fig. 2e). In a subset of *Pitx2*^{−/−} mice, the

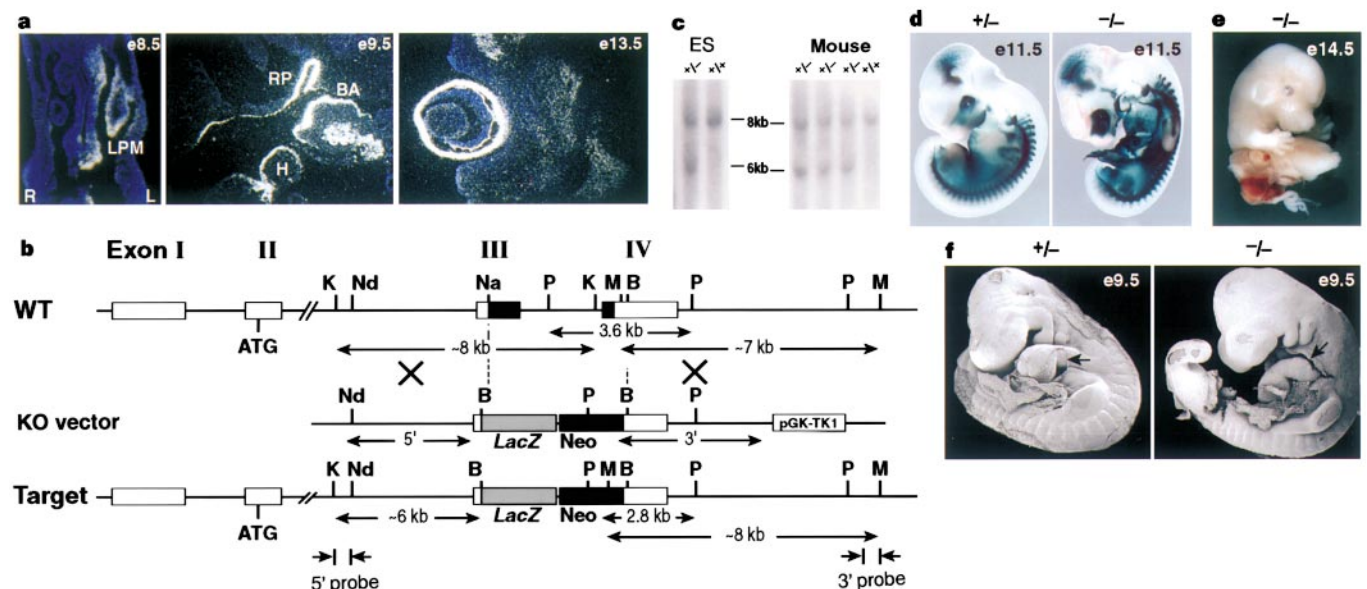


Figure 1 Targeted deletion of the *Pitx2* genomic locus. **a**, Expression of *Pitx2* in the left lateral-plate mesoderm (LPM), oral ectoderm and Rathke's pouch (RP), branchial arch (BA), heart (H) and mesoderm surrounding the eye (at E13.5). **b**, Targeted deletion of the *Pitx2* genomic locus. Appropriate 5' and 3' arms of the murine *Pitx2* gene were identified, such that the entire homeodomain and C-terminus of *Pitx2* is replaced by an in-frame *LacZ*–neomycin sequence. **c**, Homologous recombination embryonic stem (ES) cells and

mice were identified using 5' and 3' specific probes. **d**, Whole-mount *LacZ* staining of E11.5 embryos revealed a normal pattern of expression of the replaced locus. **e**, *Pitx2*^{−/−} mice exhibit a defect in closure of the ventral body wall, with externalization of thoracic and abdominal viscera. **f**, Defect in thoracic body-wall closure in E9.75 mice; arrows show marked difference in body wall closure of heterozygote and homozygote mutants.

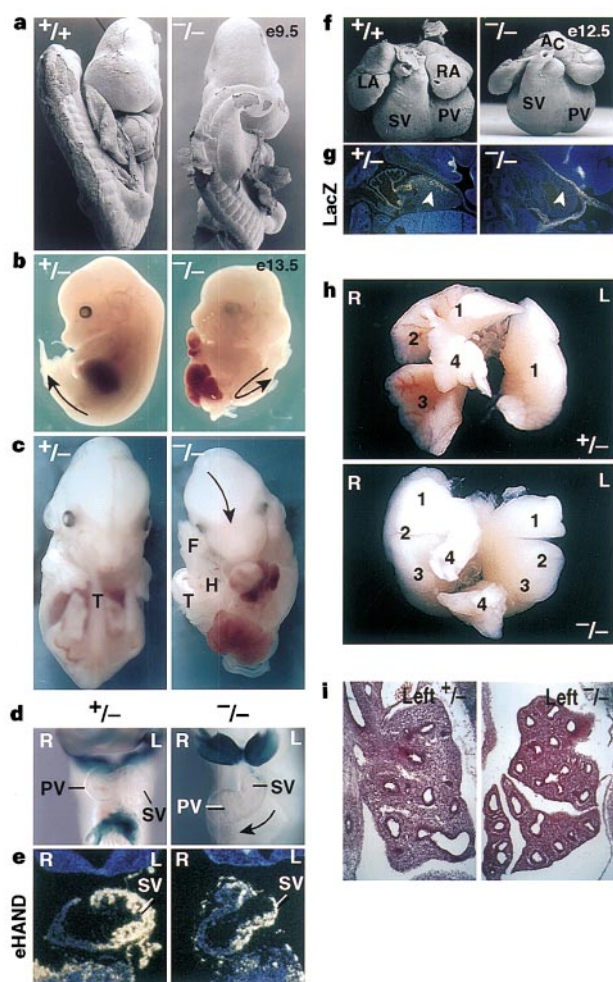


Figure 2 Role of *Pitx2* in arrest of axial turning, cardiac development and lung asymmetry. **a**, Tail turning is arrested in 50% of 36 E9–E10 *Pitx2*^{-/-} mice examined. **b**, About 30% of later embryos (E12.5–E13.5) exhibit gross dorsal, rather than ventral, axial posterior turning; ~70% (of 21 mice) have noticeable axial defects. **c**, the upper body exhibits clockwise rotation; heart (H), tail (T) and forelimb (F). **d**, Cardiac position is altered in ~48% of 21 E12.5–E13.5 *Pitx2*^{-/-} embryos. Ventral view of E9.5 embryos shows altered appearance of the looping heart in *Pitx2*^{-/-} mice with systemic ventricle (SV) in the midline and pulmonary ventricle (PV) to the right and posterior to the normal position. **e**, Expression of *eHAND* in the *Pitx2*^{-/-} mouse remains on the left in the lateral aspect of the SV. **f**, Abnormalities of later cardiac development, with varying severity of hypoplasia of PV and SV and a large common atrium (Ac). **g**, Representative sagittal section showing no detectable myocardial *LacZ* expression in *Pitx2*^{-/-} mice, despite the 2× gene dosage of *LacZ*. **h**, Right pulmonary isomerism in the *Pitx2*^{-/-} mouse. **i**, Histological sections confirm altered lobular architecture.

pulmonary ventricle extends further to the right and posteriorly than normal (Fig. 2d, e) owing to the altered biomechanics of cardiac looping. These data indicate a role for *Pitx2* in positioning the heart after the direction of cardiac looping has been determined. Later, the heart fails to septate the enlarged single atrium, and shows variable hypoplasia of the ventricles with occasional incomplete fusion (Fig. 2f, g). These events could reflect either altered haemodynamic forces or downstream *Pitx2*-dependent events affecting myocardial proliferation. Other markers of cardiac looping and development¹⁵ including *dHAND*, *Nkx2.5* and *MEF2C* are expressed normally. We do not detect *LacZ* in the myocardium after E10.5 in the *Pitx2*^{-/-} mouse, as assessed by examining serial sagittal sections (Fig. 2g); therefore, the hypoplasia of the ventricles may also, in part, reflect failure to maintain the *Pitx2*-expressing

mesodermal cell population.

The lung provides a critical test of the possibility that *Pitx2* might specify a left identity to bilaterally expressed organs that exhibit distinct left–right patterning. Murine lungs are normally characterized by a single left lobe and four right lobes. These proliferation/migration and patterning events are regulated by different members of the *Fgf* and *Bmp* families¹⁶. In *Pitx2*^{-/-} mice, both lungs invariably exhibit an apparent right pattern (Fig. 2h, i). Thus, *Pitx2* is a critical determinant of ‘left’ lung identity, and deletion of the *Pitx2* gene causes right pulmonary isomerism. Thus, the heart and lung phenotypes of *Pitx2*^{-/-} mice closely resemble those reported for deletion of the *ActRIIB* gene¹⁷.

Pitx2 is expressed over the entire period of pituitary development¹³. In the *Pitx2*^{-/-} mouse, invagination of Rathke’s pouch and direct contact with the neuroepithelium occur normally (Fig. 3a), with physiological restriction of expression of *HNF3β* (Fig. 3b) and *Shh* out of the invaginating pouch, and induction of *P-Lim/Lhx3*, *Six-1*, *Rpx/Hesx-1* and *Pitx1* (Fig. 3b, c) in response to inductive ventral diencephalic signals^{18–20}. By E10 there is a noticeable reduction of the gland (Fig. 3a). Despite the marked decrease in proliferation of the *Pitx2*^{-/-} pituitary (Fig. 3a), *Bmp2* and α GSU are initially expressed, and morphogen-induced factors including *GATA2*, *Prop-1* and *Nkx3.1* are detected at low levels, consistent with the cell number decrease (Fig. 3b). Subsequently, levels of *P-Lim/Lhx3* decrease rapidly (Fig. 3c), and by E13.5 there is a failure of organ progression, with markers of three of the ventral lineages (*Pit-1*) or rostral tip cells (*TSHβ*), as well as *Lhx4*, being undetectable, and only a few POMC-positive cells (Fig. 3b, c). By E14.5, there are only a few remaining periluminal cells. This phenotype is similar to that of the *Lhx3*^{+/-} and *Lhx4*^{-/-} (ref. 21); we find that *Pitx2* and *P-Lim/Lhx3* can synergistically activate the α GSU promoter in transfection assays. Therefore, a critical component of the *Pitx2*^{-/-} phenotype could be a failure to correctly activate target genes that require synergistic activation by *Pitx2* and *Lhx3/P-Lim*.

Tooth development exhibits defects analogous to those observed in pituitary development (Fig. 4a). The initial lamination phase of oral ectoderm²² appears to be correctly initiated on E10, and there is some condensation of the adjacent mesoderm. The initial expression of *Fgf8* during restriction to the laminating oral ectoderm is diminished, but the *Shh* signal is normal (Fig. 4b). *Bmp4* expression is switched from distal ectoderm to condensed mesenchyme (Fig. 4b), and *isl-1* from mesenchyme to oral epithelium (Fig. 4c) in the developing tooth bud in *Pitx2*^{-/-} mice on E13.5. This is followed by minimal *Msx-1* (Fig. 4c) and *Pax9* induction of condensed mesenchyme. *Bmp2* is expressed, but is not localized to the enamel knot, which fails to appear in the *Pitx2*^{-/-} tooth bud (Fig. 4b). We therefore suggest that tooth development proceeds through the initial signalling and determination phases, but that the emergence, migration and expansion of distinct cell types in the developing ectoderm fails to progress past the full bud stage²².

Pitx2^{-/-} mice also exhibit marked hypoplasia of the axial mesoderm, ventral body-wall splanchnic mesoderm and urogenital system, as well as hypoproliferation of spleen, liver, mandible, periorbital musculature and lens. The subtle decrease in expression of *Fgf8* in laminating dental epithelium (Fig. 4b) and of *Fgf18* in axial mesoderm (Fig. 4d) could be either causative or secondary to the primary molecular defect induced by *Pitx2*. We speculate that there may be a feedback loop between *Fgfs* and *Pitx2*. Indeed, organ culture of hemi-pituitaries from *Pitx2*^{+/-} mice reveals a marked decrease in expression of the *Pitx2* transcripts in response to addition of FGF2 (Fig. 4d), consistent with recent reports that *Fgf8* can inhibit *Otx2* gene expression at the midbrain/hindbrain junction²³ and *Pitx2* expression in chick²⁴.

Although *nodal* is proposed to mediate a bias in the direction of cardiac looping^{25,26}, our data indicate that *Pitx2*, a putative downstream target of *nodal*-induced pathways, regulates specific aspects of organ asymmetry and heart positioning but does not alter the

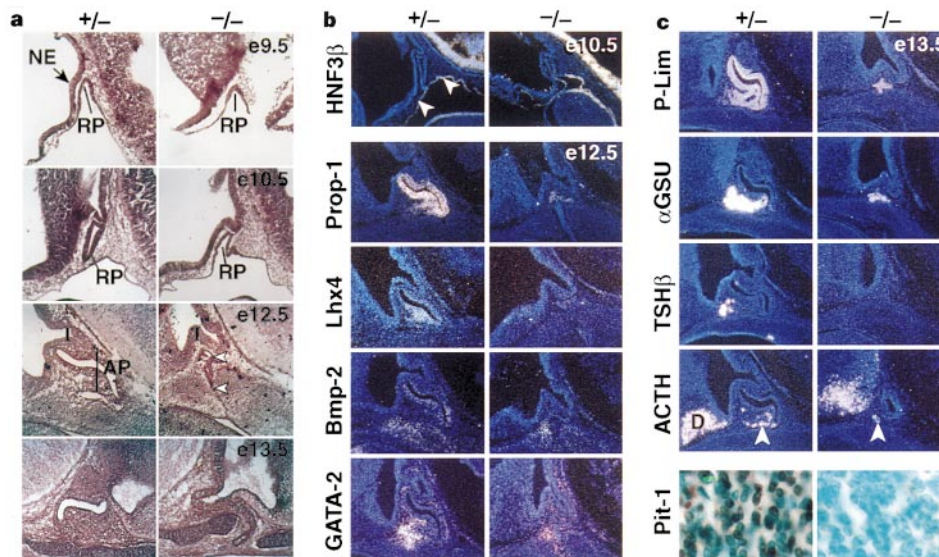


Figure 3 Role of *Pitx2* in pituitary development. **a**, Haematoxylin–eosin-stained sagittal sections of the pituitary gland from E9.5–E13.5. Note normal formation of Rathke's pouch (RP), with decreased cell content by E10.5. The gland (see arrowheads) is hypoplastic by E12.5 and beyond. **b**, **c**, *In situ* hybridization of E9.5 Rathke's pouch using a

series of probes including *HNF3β*, *Prop-1*, *GATA-2*, *Bmp2*, *P-Lim*, *αGSU*, *TSHβ*, *Pit-1* and *POMC* probes. AP, anterior pituitary; D, ventral diencephalon; NE, neuropithelium; I, infundibulum.

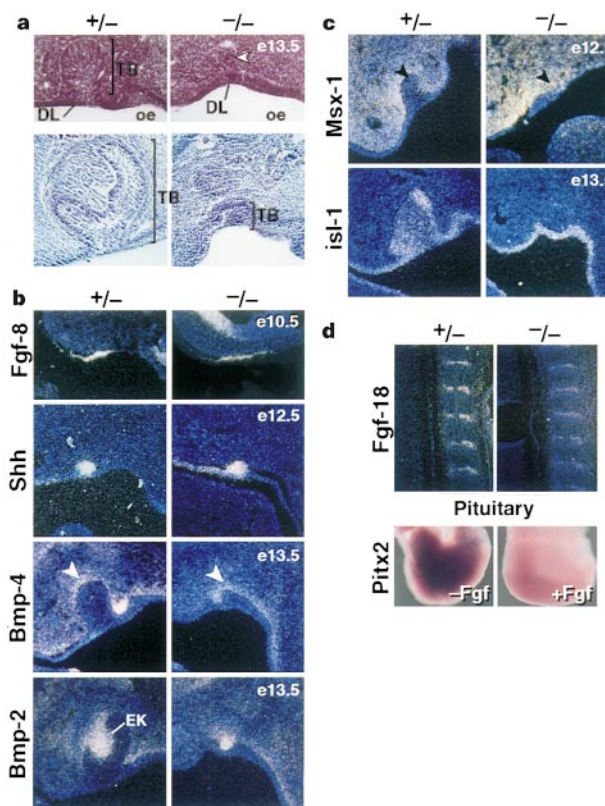


Figure 4 Role of *Pitx2* in tooth development. **a**, The initiation steps of tooth formation occur with the appearance of dental lamination (DL) from the oral ectoderm (oe). The tooth bud (TB) is evident in heterozygous mice by E12.5, but does not progress beyond initiation in *Pitx2*^{-/-} mice. By E14.5, the cap stage is evident in the tooth buds of *Pitx2*^{+/-} mice; there is only some minimal condensation of overlying mesenchyme and proliferation of oral ectoderm in *Pitx2*^{-/-} mice. **b**, **c**, Expression of genes encoding signalling molecules in *Pitx2*^{-/-} mice. *Shh* is expressed in the initiating region, and there is initial appearance of *Bmp4* in the mutant tooth bud and shift of the expression to the condensing mesenchyme.

Bmp2 is expressed, but the enamel knot (EK) never appears. There is diminished *Fgf8* expression in the regions of initial distal proliferation on E10.5–E11.0, and *Fgf8* normally disappears in both heterozygous and homozygous mutants by E11.5. **d**, Expression of *Fgf18* in *Pitx2*^{+/-} and *-/-* E12.5 axial mesoderm, with apparent lower *Fgf18* expression in the *Pitx2*^{-/-} mice. FGF2 induces a dramatic decrease in *Pitx2* gene expression in adult hemi-pituitary from *Pitx2*^{+/-} mice cultured in the presence or absence of FGF2 (3×10^{-7} M) for 14 h in serum-free defined medium.

direction of cardiac looping. The similarity of the cardiac and pulmonary phenotypes of *Pitx2*^{-/-} and *ActRIIB*^{-/-} (ref. 17) indicates that *Pitx2* is the critical downstream target of *ActRIIB*, a putative Nodal receptor, mediating effects on both cardiac positioning and pulmonary isomerism. The *Pitx2* phenotype extends to tissues other than those affected by *ActRIIB*, indicating that *Pitx2* gene activation may be controlled separately in these tissues. *Pitx2* is thus, to our knowledge, the first transcription factor that can be considered to be a critical component in mediating the proliferative/motility events of the lateral-plate mesoderm that underlie ventral body-wall closure and 'leftness' in lung patterning and organ placement, as well as serving critical later roles in organogenesis. □

Methods

Generation of *Pitx2*-knockout mice.

Genomic clones encompassing the entire *Pitx2* transcript were obtained from the 129/SvJ mouse genomic library (Stratagene). The 5'-arm is a 5.0-kilobase (kb) *NdeI*-*Ngo*MI fragment bordering the DNA-binding domain and the 3'-arm is a 4.5-kb *Bam*HI-*Sal*I 3'-end fragment that includes the entire 3' untranslated region. The region from the homeodomain to the C-terminus of *Pitx2* is replaced by the *LacZ*/*neomycin* sequences, driven by the *Pitx2* promoter. R1 embryonic stem cells were grown and selected with G418 and gancyclovir, and Southern blot analysis was carried out using a 5'-external (1.6-kb) probe that hybridizes to a 8-kb *Kpn*I wild-type and a 6-kb *Kpn*I/*Bam*HI recombinant band. We confirmed genotyping of heterozygotes and homozygotes using a 3'-internal (0.6-kb) probe that recognizes a 3.6-kb *Pst*I fragment in the wild-type and a 2.8-kb *Pst*I fragment in the recombinant alleles.

In situ hybridization, whole-mount hybridization and *LacZ* staining.

Isolation, fixation, and hybridization with ³⁵S-labelled antisense RNA probes and exposure were done as described¹. For whole-mount *in situ* hybridization studies, embryos were briefly fixed in 4% paraformaldehyde followed by methanol and hydrogen peroxide washes and proteinase-K treatment as described¹. All probes were as described^{1,2}, or derived by PCR and sequenced.

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Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14

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Urotensin-II (U-II) is a vasoactive 'somatostatin-like' cyclic peptide which was originally isolated from fish spinal cords^{1,2}, and which has recently been cloned from man³. Here we describe the identification of an orphan human G-protein-coupled receptor homologous to rat GPR14 (refs 4, 5) and expressed predominantly in cardiovascular tissue, which functions as a U-II receptor. Goby and human U-II bind to recombinant human GPR14 with high affinity, and the binding is functionally coupled to calcium mobilization. Human U-II is found within both vascular and cardiac tissue (including coronary atheroma) and effectively constricts isolated arteries from non-human primates. The potency of vasoconstriction of U-II is an order of magnitude greater than that of endothelin-1, making human U-II the most potent mammalian vasoconstrictor

identified so far. *In vivo*, human U-II markedly increases total peripheral resistance in anaesthetized non-human primates, a response associated with profound cardiac contractile dysfunction. Furthermore, as U-II immunoreactivity is also found within central nervous system and endocrine tissues, it may have additional activities.

In an effort to identify novel G-protein-coupled receptors (GPCRs), we probed a human genomic library with rat GPR14 (SEN14)^{4,5}, an orphan GPCR similar to somatostatin receptors. We isolated a 9-kilobase (kb) genomic clone which encoded a 389-residue human GPCR (75% identity to rat GPR14; Fig. 1). GPR14 messenger RNA was most abundant in the heart and pancreas (no expression was detected in 15 discrete brain regions; see Supplementary Information); however, using quantitative polymerase chain reaction with reverse transcription (RT-PCR), low levels of transcript were detectable in thalamus, superior occipital gyrus and substantia nigra. Additional RT-PCR revealed expression in human cardiac (atria and ventricle) and arterial (aorta) tissue, endothelial (coronary artery and umbilical vein) cells and smooth muscle (coronary artery and aortic) cell lines, but not in vena cava nor in a venous (renal vein) smooth muscle cell line.

Human GPR14 was subcloned into a mammalian expression vector and transfected into HEK-293 cells. In a 'reverse pharmacological approach'^{6,7}, the cells were used to search for a ligand activating GPR14 using a calcium-mobilization assay. Using a fluorescent imaging plate reader (FLIPR), transiently transfected cells were exposed to over 700 known and putative GPCR agonists. We detected a robust calcium-mobilization response in cells transfected with either rat or human GPR14 only upon challenge with goby U-II (effector concentration for half maximum response

(EC₅₀) of 0.78 ± 0.18 and 0.47 ± 0.14 nM, respectively; Fig. 2a). This response was highly selective for goby U-II and was not induced by any of the other ligands we tested. Cells transfected with expression vector alone, or vectors encoding other orphan GPCRs such as GPR7 or GPR8, which are also homologous to the somatostatin receptors⁸, did not respond to goby U-II.

A human GPCR that selectively responded to a non-mammalian neuropeptide indicated the possible existence of a human orthologue. A search of GenBank using the carp U-II sequence⁹ yielded a match to a human expressed sequence tag (EST) with 25% identity to the fish sequence. Using this EST, we isolated a 688-base pair (bp) complementary DNA encoding human U-II (Fig. 3). The deduced propeptide sequence contained an amino-terminal signal sequence (cleaved at residue 17) and three putative polybasic proteolytic cleavage sites (Fig. 3), but varied at the amino terminus from the human U-II propeptide described subsequently in ref. 3, probably as a result of alternative splicing. The mature sequence (ETPDCFW-KYCV) was identical to human U-II (ref. 3), and it retained the cyclic hexapeptide sequence (CFWKYC) that is absolutely conserved across species^{1,2}. Expression of the U-II transcript was restricted to the spinal cord and medulla oblongata (see Supplementary Information), consistent with previous reports in fish, frogs and humans¹⁻³.

Human U-II induced concentration-dependent increases in intracellular calcium in a HEK-293 cell line expressing human GPR14 (EC₅₀ = 0.62 ± 0.17 nM, $n = 6$; Fig. 2b). ¹²⁵I-labelled goby U-II bound saturably and with high affinity to membranes prepared from these cells (Fig. 2c; $K_d = 0.43 \pm 0.01$ nM; $B_{max} = 447 \pm 58$ fmol mg⁻¹ protein; $n = 3$). Goby and human U-II displaced the radioligand with comparable affinities

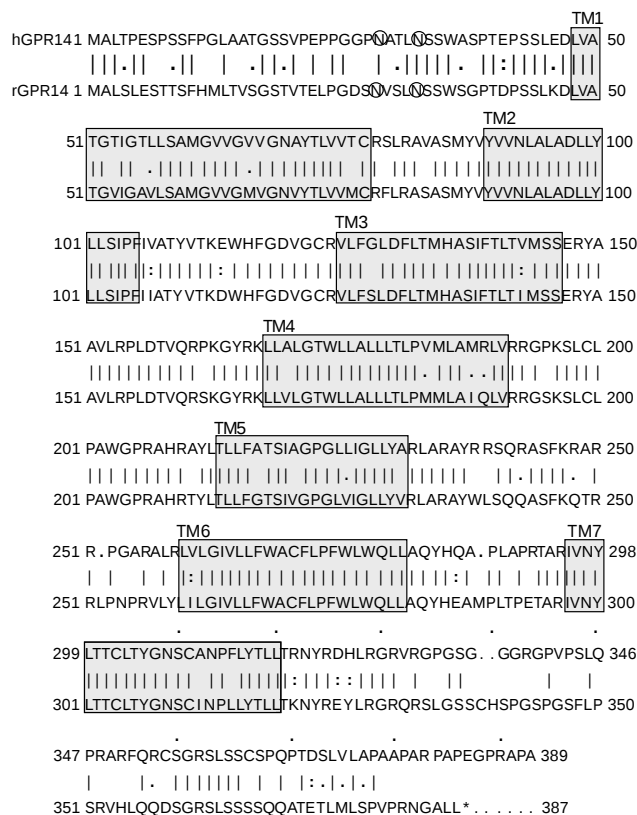


Figure 1 Alignment of rat (r) and human (h) GPR14 polypeptide sequences. The predicted seven transmembrane (TM) spanning regions are shaded and potential N-linked glycosylation sites are circles. GenBank accession number of human GPR14 is AF140631.

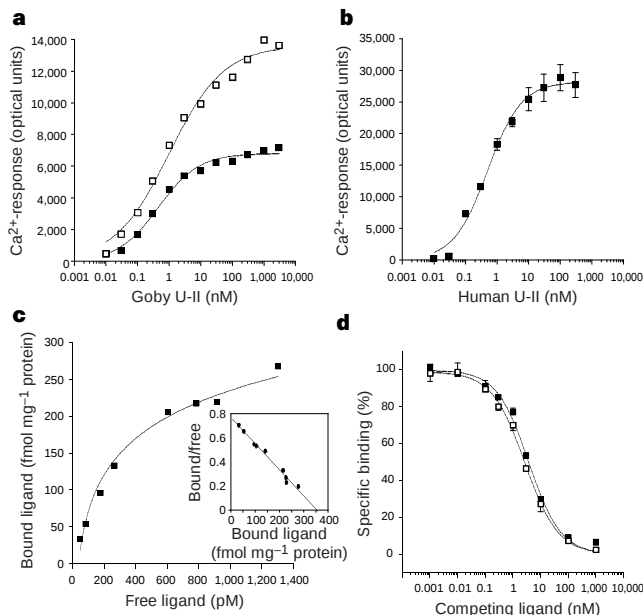


Figure 2 GPR14 is a U-II receptor. **a**, Goby U-II-induced Ca²⁺-mobilization (FLIPR) in HEK-293 cells transiently transfected with rat (filled squares) or human GPR14 (open squares). **b**, Ca²⁺-mobilization response to human U-II in clonal HEK-293 cell lines stably transfected with human GPR14. **c**, Saturation binding isotherm for ¹²⁵I-labelled goby U-II in cell membranes from HEK-293 cells stably transfected with human GPR14 (inset, Scatchard analysis). **d**, Competition binding curves for human (filled squares) and goby U-II (open squares) with human GPR14.

1	TGCCGGTGCCCTCATG6GCAGGACTAGGCCGAAAACCTCCCAGATTGTCAITTCACG66	69
61	ATGGCAGCCCTAAACAGCAGCTGGCAACTCATCTACTCACTCAAGAAATGAAAAATG	120
121	GAACCAACGATTTATGCTATGTGCTCTGCGTCATCTGTCTGGAGCATATAATCAACG	180
181	ETNVFHLMLCVSTARHTKST	240
241	TCTTTTGTCTGG6CACTTCAACTCATCTCAAG6CTTCTTTAATCATGATTTATTG	300
301	SLCFCGHFNSYSPSLIHDGL	360
361	CTGGAAGATCTCTTCAACTCTCAGCAGCTCATGAAGACGGCGCTTAACTCGGAGAG	420
421	LIEISFLDSAPHEDARLTPEE	480
481	CTAGAAGAGCTTCCCTTTACAGATAGCTCGCAGAGAGCTGGGTGCAGAAAGAGGGGAT	540
541	LERASALGQILPELMLGAERGD	600
601	ATTCTCAGGAAGCAGACTCAAGTACCAACTTTTTAACCCAGAGGAAATTTGAGAAG	660
661	ITL R K D AKDSNTINFNPRGN L R K	
1	TTCAGGATTTCTGTGACAGAGCTCTAACTTTTACTGAGTCATCTTTGGCCAGAACT	
1	FQDDFSGQDPNILLSHLLARL	
1	TGGAACCCATCAAGAAAGCTGAGACTCTGATTGCTCTGGAAGATCTGTTCTGAAGT	
1	W K P Y K R K E T P D C F W K Y C V	
1	GAATTAAGCATCTTTAGTCACTCAGAAACCCACTCTTGAAGATTGAAAAATAACACA	
1	ATGCTTGATTTGTTAAACCAAGCTGGGAGAAACATGGCAGAACTACACCTGTTCTATTGTA	
1	CTGGAATTAATAATCTCTATGTTTTCG	688

Figure 3 Nucleotide and deduced peptide sequence of human U-II propeptide cDNA. Putative polybasic cleavage sites are shown in bold and underlined. The putative mature human U-II is shown in italics. GenBank accession number is AF140630.

Table 1 Relative contractile potency of human U-II in non-human primates

Vessel	Human urotensin-II -log[EC ₅₀]	Endothelin-1 -log[EC ₅₀]	Relative potency
Arterial tissue			
Left anterior descending coronary	9.39 ± 0.40	8.20 ± 0.32*	15
Left circumflex coronary	9.56 ± 0.05	n.d.	
Mesenteric	9.35 ± 0.26	8.24 ± 0.28*	13
Pulmonary	9.29 ± 0.16	7.84 ± 0.06***	28
Renal	9.59 ± 0.36	8.83 ± 0.24	6
Proximal descending thoracic aorta	8.96 ± 0.24	n.d.	
Distal descending thoracic aorta	9.06 ± 0.22	n.d.	
Distal abdominal aorta	9.37 ± 0.13	n.d.	
Basilar	10.00 ± 0.16	n.d.	
Common carotid	9.16 ± 0.08	n.d.	
Internal mammary	9.30 ± 0.08	n.d.	
Venous tissue			
Portal vein	<7.00	7.00 ± 0.77	
Jugular vein	<7.00	8.88 ± 0.21	

All values are mean $\pm$ s.e.m. ($n = 3-4$). Statistical comparisons made by analysis of variance (post-hoc Fisher's least-squares difference): * $P < 0.05$ and *** $P < 0.001$. n.d., not determined.

($K_i = 2.06 \pm 0.29$ and 1.99 ± 0.23 nM, respectively; Fig. 2d). Hill coefficients for goby and human U-II approximated to unity ($n_H = 0.8 \pm 0.1$ and 0.9 ± 0.1 , respectively), indicative of interactions with a single, homogeneous population of binding sites. Binding sites for fish U-II are found in rat arterial membranes, although receptor density is extremely low ($2\text{--}20$ fmol mg^{-1} protein¹⁰). We have made similar observations in rat cardiac membranes ($K_d = 0.35$ nM; $B_{\max} = 3.8$ fmol mg^{-1} protein). Although U-II and somatostatin are structurally similar¹¹, human GPR14 functioned as a U-II-selective receptor: $1\text{ }\mu\text{M}$ concentrations of somatostatin₍₁₋₁₄₎, urotensin-I, vasopressin, angiotensin-II, MCH, salmon calcitonin and NPY did not compete for binding; neither did they stimulate calcium mobilization in HEK-293 cells expressing human GPR14.

Immunohistochemical evaluation of monkey and human tissue showed (see Supplementary Information) that the vasculature contained U-II-like immunoreactivity. Diffuse immunostaining was observed in human cardiomyocytes and coronary atherosclerotic plaque, where the staining was intense within the lipid-laden smooth muscle/macrophage-rich regions. We noted additional immunoreactivity within spinal cord ventral horn motor neurons and acinar cells lining the thyroid follicles.

Human and goby U-II induced potent and efficacious contractions in the isolated thoracic aorta of rat ($-\log[\text{EC}_{50}] = 9.09 \pm 0.19$, $n = 13$; $-\log[\text{EC}_{50}] = 9.22 \pm 0.18$, $n = 15$, respectively; Fig. 4a). Human U-II was significantly more potent ($P < 0.001$) than endothelin-1 (ET-1), noradrenaline and serotonin ($-\log[\text{EC}_{50}] = 7.90 \pm 0.11$, 7.58 ± 0.11 and 6.27 ± 0.12 , respectively; $n = 11, 13$ and 4 , respectively; Fig. 4a). Thus, U-II is the most potent human vasoconstrictor peptide identified so far (16-fold

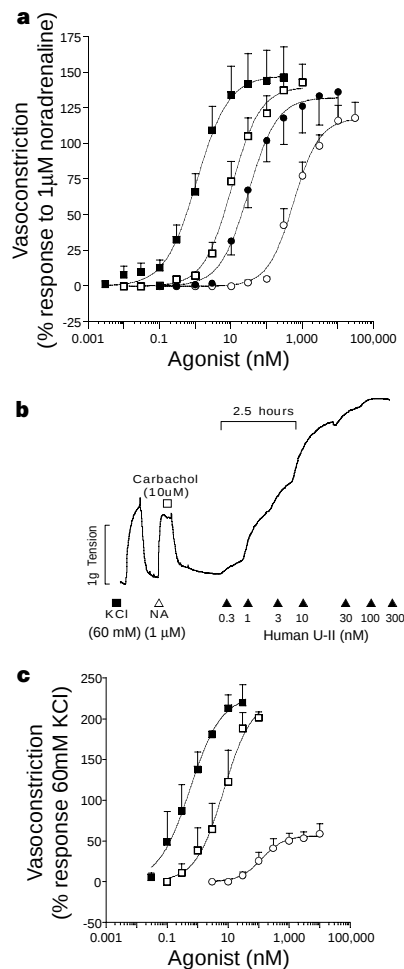


Figure 4 Human U-II is a potent vasoconstrictor. **a**, Concentration-dependent contraction of rat isolated aorta by human U-II (filled squares; $n = 13$), ET-1 (open squares; $n = 11$), noradrenaline (filled circles; $n = 13$) and serotonin (open circles; $n = 4$). **b**, Trace showing concentration-dependent contraction of the primate isolated aorta by human U-II (filled triangles). Responses to standard concentrations of KCl (filled square) and noradrenaline (open triangle) are shown (successful endothelial denudation is shown by the lack of carbachol-induced vasodilation; open square). **c**, Concentration-dependent contraction of the cynomolgus monkey isolated coronary artery ($n = 4$) in response to human U-II (filled squares), ET-1 (open squares) and serotonin (open circles).

more potent than ET-1 in rat).

The vasoconstrictor activity of human U-II was limited to the thoracic aorta in rat: abdominal aorta or femoral and renal arteries did not contract (radioligand binding sites were undetectable in vessels distal to the aortic arch)¹⁰. Consequently, U-II lacks systemic pressor activity in anaesthetized rats upon intravenous (i.v.) administration^{12,13}. In contrast, however, human U-II caused contraction in all non-human primate arterial vessels studied, including both elastic and muscular arteries (Fig. 4b, c). EC₅₀ values were subnanomolar, making human U-II 6–28-fold more potent than ET-1 (Table 1). Unlike ET-1, however, the contractile actions were restricted to the arterial side of the vasculature, consistent with the differential expression of human GPR14 determined by PCR. Thus, the contractile profile of U-II is species-dependent and U-II exhibits an ‘anatomically diverse’ contractile profile in the arterial vasculature of the primate.

Following this striking *in vitro* profile, we carried out haemodynamic evaluation in anaesthetized monkeys. Systemic administra-

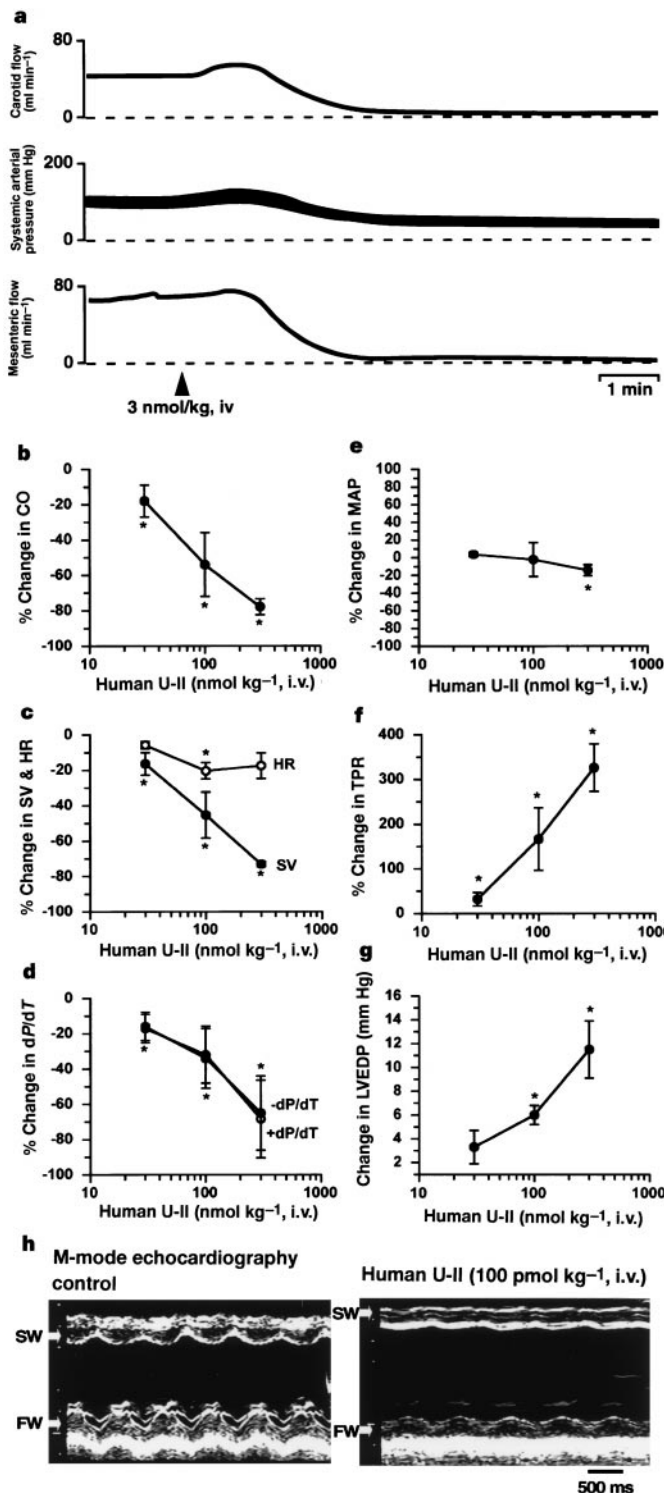


Figure 5 Human U-II produces cardiovascular dysfunction in the anaesthetized monkey. **a**, Fatal circulatory collapse (precipitous falls in systemic pressure and regional flows) induced by bolus i.v. human U-II. Alterations in cardiac output (CO) (**b**), heart rate (HR, open circles) and stroke volume (SV, filled circles) (**c**), myocardial contractility (dP/dT) (**d**), mean arterial pressure (MAP) (**e**), total peripheral resistance (TPR) (**f**) and left ventricular end diastolic pressure (LVEDP) (**g**). *, $P < 0.05$ relative to vehicle ($n = 5$). **h**, M-mode echocardiographic images of the left ventricle before and 7 min after 100 pmol kg⁻¹, i.v. human U-II show severe attenuation of septal (SW) and free wall (FW) motion.

tion of human U-II elicited a complex dose-dependent haemodynamic response that culminated in severe myocardial depression and fatal circulatory collapse (Fig. 5a). Unusually, a 300 pmol kg⁻¹ dose of U-II, which induces a 300% increase in total peripheral resistance (indicative of systemic vasoconstriction), did not cause concomitant systemic hypertension. The ability of U-II to induce marked systemic vasoconstriction while also causing catastrophic myocardial contractile dysfunction (for example, 80% decrease in dP/dT and stroke volume) differentiates it from other peptides such as ET-1 and angiotensin-II. For example, 1 nmol kg⁻¹ ET-1 i.v. (sufficient to elevate total peripheral resistance) is accompanied, not by cardiovascular collapse and systemic hypotension, but rather by a sustained (≤ 1 h) 60 mm Hg increase in arterial pressure¹⁴.

The primate responses were clearly different from those reported in rats^{12,13}. At doses < 30 pmol kg⁻¹ i.v., human U-II slightly increased cardiac output and reduced regional vascular resistance, with little or no change in arterial blood pressure. In contrast, at doses ≥ 30 pmol kg⁻¹, human U-II decreased myocardial function and increased vascular resistance. Haemodynamic and echocardiographic analysis demonstrated a dose-dependent reduction in cardiac output (Fig. 5b), the result of mild bradycardia and severely attenuated stroke volume (Fig. 5c). Myocardial contractility was severely depressed, as seen by reductions in dP/dT (Fig. 5d). Despite a marked decrease in cardiac output, sub-lethal doses reduced mean arterial pressure only moderately (Fig. 5e) because of a threefold increase in total peripheral resistance (Fig. 5f). Decreases in regional blood flows and a dose-dependent increase in left ventricular end diastolic pressure (Fig. 5g) indicated systemic vasoconstriction. The severe loss of myocardial contractility was shown by M-mode echocardiography (Fig. 5h) in which grossly attenuated septal and free-wall motion was evident (ultimately, this effect was terminal at doses ranging from 100–3,000 pmol kg⁻¹). Systemic human U-II administration produced ST segment changes in the electrocardiogram typical of those seen during myocardial ischaemia.

In summary, we have isolated a human GPCR that exhibits the pharmacological characteristics of a U-II receptor. Cells expressing this receptor exhibit high-affinity binding sites for, and respond to, both goby and human U-II. Furthermore, we have demonstrated that U-II is the most potent vasoconstrictor identified so far. Systemic administration is associated with profound cardiovascular collapse. As human U-II-like immunoreactivity is found within cardiac and vascular tissue (including coronary atheroma), this peptide may influence cardiovascular homeostasis and pathology (for example, in ischaemic heart disease and congestive heart failure). Finally, the detection of immunoreactivity within spinal cord and thyroid tissue raises the possibility that U-II may also influence central nervous system and endocrine function in man. □

Methods

Cloning

A human genomic placental library (Stratagene) was screened with a ³²P-labelled 1.2-kb PCR-derived clone encoding rat GPR14 (ref. 4), and we identified a positive phage plaque. Two kilobases of insert was sequenced and determined to be homologous to rat GPR14. Human GPR14 was isolated by PCR and subcloned into pCDN (ref. 15). HEK-293 cells were transiently or stably transfected using Lipofectamine Plus (Life Technologies). Using the published fish cDNA sequence², a search of the GenEMBL EST database identified a human cDNA (AA535545) encoding a putative U-II orthologue. After, 5'-rapid amplification of cDNA ends (RACE) (placenta and Raji Marathon Ready cDNAs; Clontech), we sequenced and subcloned the resultant products into pCR2.1 (Invitrogen).

Peptide synthesis

Human U-II peptide resin was synthesized (Milipore/Bioscience 9600 synthesizer), cleaved and de-protected before purification by reverse-phase HPLC¹⁶. All other peptides were from Bachem.

Calcium mobilization

We used a microtitre-plate-based calcium-mobilization FLIPR assay, as described previously¹⁷, for the functional identification of ligands that activate HEK-293 cells expressing recombinant GPR14. Ligands we tested included micromolar concentrations of all known mammalian neuropeptides and $\geq 10 \mu\text{M}$ concentrations of all known steroid, lipid and amine transmitters.

Radioligand binding

Membranes from HEK-293 cells stably transfected with human GPR14 were pre-coupled to wheatgerm-agglutinin-coated SPA beads (Amersham). Using mono-iodinated goby U-II (¹²⁵I-labelled Tyr¹⁰, chloramine T, 2000 Ci mmol⁻¹; 5 mM MgCl₂, 0.1% BSA, 20 mM Tris-HCl pH 7.4, 25 μg protein; 25 °C), equilibrium binding was measured in 96-well plates (Wallac) after 45 min¹⁸. Non-specific binding, determined using 1 μM unlabelled U-II, was $\sim 10\%$ of total binding. There was no specific binding to mock-transfected HEK-293 membranes. We determined protein using biochonic acid (Pierce). Kinetic analysis was by nonlinear least square fitting (GraphPad).

Isolated blood vessel studies

Isolated arterial rings from male Sprague-Dawley rats (400 g) and cynomolgus monkeys (*Macaca fascicularis*) (5 kg) were suspended in organ baths containing Krebs (37 °C; 95% O₂; 5% CO₂)¹⁹. Changes in isometric force were recorded under optimal resting tension. Cumulative agonist concentration-response curves were normalized to 60 mM KCl or 1 μM noradrenaline and fitted to a logistic equation¹⁹. Unless stated otherwise, responses were measured in the presence of 10 μM indomethacin using vessels denuded by rubbing (confirmed as a loss of dilator response to 10 μM carbachol).

Haemodynamics and echocardiography

All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (DHSS publication NIH 85-23). Adult male cynomolgus monkeys (5–7 kg) were treated with atropine sulphate (25 μg kg⁻¹ subcutaneous) and anaesthetized with ketamine (10 mg kg⁻¹ intramuscular). After endotracheal intubation, anaesthesia was maintained with 1–3% isoflurane. We prepared the animals for monitoring of systemic haemodynamics. Echocardiography (Doppler, M-mode and 2-D, ATL5000) was carried out at 2–3 min intervals. Vehicle administration (0.9% NaCl) was followed by the cumulative administration of human U-II.

Statistics

Values are expressed as mean $\pm$ s.e.m. *n* is the number of individual observations made in a particular group. Statistical comparisons were made by one-way analysis of variance (Fisher's protected least-squares difference) and differences considered significant where *P* < 0.05.

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A kinase-regulated PDZ-domain interaction controls endocytic sorting of the β 2-adrenergic receptor

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A fundamental question in cell biology is how membrane proteins are sorted in the endocytic pathway. The sorting of internalized β 2-adrenergic receptors between recycling endosomes and lysosomes is responsible for opposite effects on signal transduction and is regulated by physiological stimuli^{1,2}. Here we describe a mechanism that controls this sorting operation, which is mediated by a family of conserved protein-interaction modules called PDZ domains³. The phosphoprotein EBP50 (for ezrin-radixin-moesin (ERM)-binding phosphoprotein-50)⁴ binds to the cytoplasmic tail of the β 2-adrenergic receptor through a PDZ domain and to the cortical actin cytoskeleton through an ERM-binding domain. Disrupting the interaction of EBP50 with either domain or depolymerization of the actin cytoskeleton itself causes missorting of endocytosed β 2-adrenergic receptors but does not affect the recycling of transferrin receptors. A serine residue at position 411 in the tail of the β 2-adrenergic receptor is a substrate for phosphorylation by GRK-5 (for G-protein-coupled-receptor kinase-5) (ref. 5) and is required for interaction with EBP50 and for proper recycling of the receptor. Our results identify a new role for PDZ-domain-mediated protein interactions and for the actin cytoskeleton in endocytic sorting, and suggest a mechanism by which GRK-mediated phosphorylation could regulate membrane trafficking of G-protein-coupled receptors after endocytosis.

Surface-biotinylated β_2 -adrenergic receptors (β_2 -ARs) expressed in human embryonic kidney (HEK) 293 cells exhibited no detectable degradation when incubated in the presence of the agonist isoprenaline for 4 h (Fig. 1a, β_2 -AR), consistent with the rapid and efficient recycling of internalized β_2 -ARs to the plasma membrane in this cell type^{6,7}. However, truncation of the distal portion of the cytoplasmic tail caused pronounced, ligand-induced degradation (Fig. 1a, β_2 -AR_t). Addition of various residues to the full-length tail, including a single alanine residue (β_2 -AR-Ala) also caused ligand-induced degradation. The $t_{1/2}$ for ligand-induced degradation of the wild-type receptor protein was >9 h. In contrast, all mutations of the distal β_2 -AR tail caused ligand-induced degradation that was nearly complete ($\geq 75\%$) within 4 h (Fig. 1b).

To investigate the role of the distal tail in β_2 -AR trafficking, we visualized the membrane trafficking of antibody-labelled receptors⁶ using fluorescence microscopy. Both the wild-type β_2 -AR and β_2 -AR-Ala endocytosed rapidly following activation by isoprenaline, as indicated by the translocation of antibody-labelled receptors from the plasma membrane to endocytic vesicles (Fig. 2Aa and e). After removal of the agonist, wild-type β_2 -AR recycled efficiently to the plasma membrane (Fig. 2Ab–d) whereas β_2 -AR-Ala did not (Fig. 2Af–h). Quantification by biotinylation (Fig. 2B) and flow cytometry (data not shown) confirmed that wild-type β_2 -ARs recycled efficiently within ≤ 30 min after agonist removal, whereas recycling of β_2 -AR-Ala was significantly ($\geq 50\%$) inhibited.

Many vesicles containing internalized β_2 -AR-Ala colocalized with membrane markers of late endosomes and lysosomes, as seen by confocal microscopy (data not shown). The lysosomotropic agents chloroquine and NH_4Cl , as well as the lysosomal protease inhibitor leupeptin, significantly inhibit agonist-induced degradation of β_2 -AR-Ala (Fig. 2C). These results indicate that mutation of the distal cytoplasmic tail inhibits efficient recycling and causes missorting of internalized receptors to lysosomes.

To identify candidate proteins involved in this sorting operation, we searched for proteins that bind to the cytoplasmic tail of the wild-type β_2 -AR but not to β_2 -AR-Ala. A predominant immunoreactive protein present in HEK293 cell lysates that bound specifically to the wild-type β_2 -AR tail was EBP50, as identified using an antibody recognizing members of the EBP50/NHERF/E3KARP protein family⁴ (Fig. 3A). EBP50 is a human homologue of rabbit

NHERF⁸ and a relative of E3KARP⁹. NHERF is a Na^+/H^+ -exchanger regulator factor^{8,9} that functions in β_2 -AR-mediated signal transduction at the plasma membrane¹⁰. NHERF and E3KARP bind to the cytoplasmic tail of the β_2 -AR by an amino-terminal PDZ domain¹¹. We confirmed, by affinity chromatography, that the homologous PDZ domain from EBP50 bound to the cytoplasmic tail of the β_2 -AR but not β_2 -AR-Ala (data not shown). Endogenous EBP50 co-immunoprecipitated with full-length but not mutant β_2 -ARs, indicating that this protein interaction may occur *in vivo* (Fig. 3B).

EBP50 can link membrane proteins with the cortical actin cytoskeleton through association with ERM proteins⁴. If such a linker function is required for endocytic sorting of the β_2 -AR, disruption of the EBP50–ERM interaction may have a similar effect on sorting of the wild-type β_2 -AR to that of disruption of the β_2 -AR–EBP50 interaction caused by mutation of the receptor tail. We constructed a truncated mutant form of EBP50 (EBP50 Δ) that lacks the ERM-binding domain¹² but retains the PDZ domain that is required for specific interaction with the β_2 -AR. This was confirmed by affinity chromatography, showing binding of EBP50 Δ to the cytoplasmic tail of the wild-type β_2 -AR but not to the β_2 -AR-Ala (data not shown). Overexpression of EBP50 Δ in stably transfected cells caused missorting and enhanced lysosomal degradation of the full-length wild-type β_2 -AR after agonist stimulation (Fig. 3C). Overexpression of the wild-type EBP50 protein at similar levels (determined by immunoblotting) had a much smaller effect on β_2 -AR degradation (Fig. 3C). In contrast, neither EBP50 Δ nor overexpressed wild-type EBP50 affected the recycling of transferrin receptors analysed in the same cell extracts (not shown). These results indicate that the proper sorting of internalized β_2 -ARs requires EBP50 to interact, through distinct domains, with both the β_2 -AR cytoplasmic tail and ERM proteins.

Interaction with ERM-family proteins links EBP50 to the cortical actin cytoskeleton⁴, indicating that the actin cytoskeleton may be

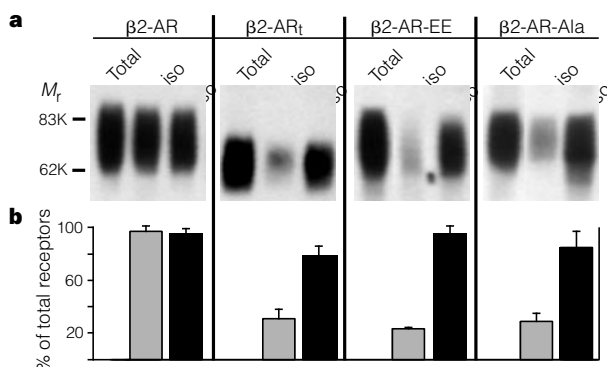


Figure 1 β_2 -AR mutants are degraded. **a**, Mutation of the β_2 -AR cytoplasmic tail results in receptor degradation. The biotinylation assay of receptor degradation was used to compare agonist-induced degradation of wild-type β_2 -AR (β_2 -AR) and C-terminal truncation (β_2 -AR_t) or addition (β_2 -AR-EE and β_2 -AR-Ala) mutations. M_r , relative molecular mass **b**, Quantification of receptor degradation by densitometric scanning of streptavidin overlays. Bars indicate mean biotinylated receptor protein recovered 4 h after biotinylation. Error bars represent s.d.

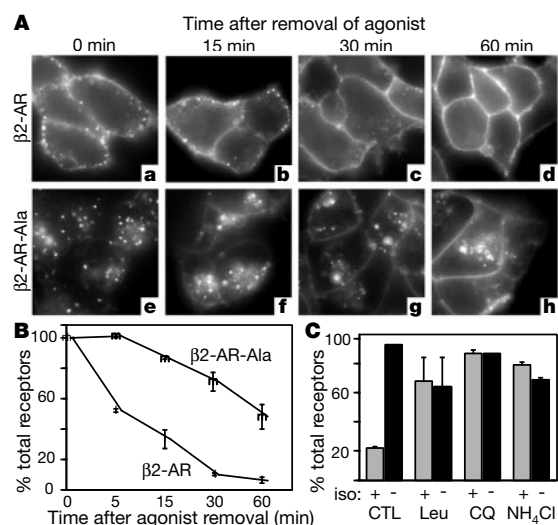


Figure 2 Missorting of mutant β_2 -ARs to lysosomes. **A**, Recycling of β_2 -AR-Ala is inhibited. Agonist-internalized β_2 -ARs present in endocytic vesicles (**a**) returned to the plasma membrane following agonist removal (**b–d**), whereas internalized β_2 -AR-Ala (**e**) remained in endocytic vesicles (**f–h**). **B**, Inefficient recycling of β_2 -AR-Ala measured using a biotinylation recycling assay. Efficient recycling of wild-type β_2 -AR but not β_2 -AR-Ala was observed by glutathione-resistance. **C**, Lysosomal degradation of β_2 -AR-Ala. Degradation of biotinylation β_2 -AR-Ala was significantly inhibited in cells treated with leupeptin (Leu), chloroquine (CQ) or NH_4Cl . Ctl, control. Error bars represent s.d.

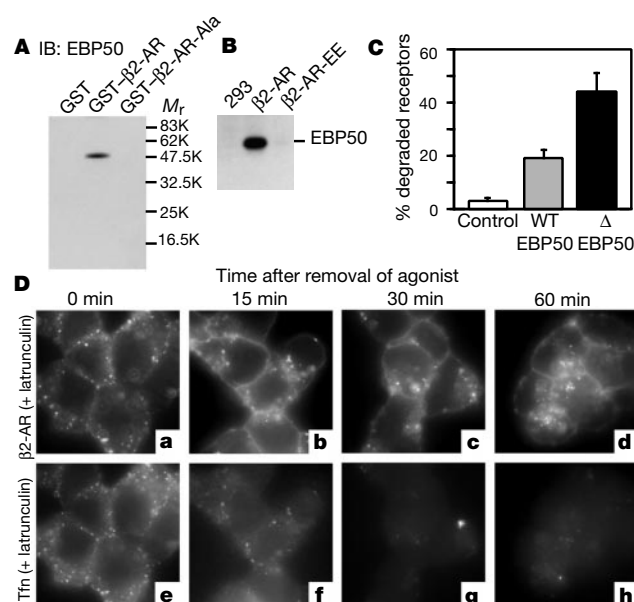


Figure 3 Role of EBP50 and actin in β 2-AR sorting. **A**, EBP50 binds to the wild-type β 2-AR cytoplasmic tail. Binding of EBP50 from HEK293 extracts to wild-type but not β 2-AR-Ala tail fusion proteins. **B**, EBP50 associates with β 2-AR *in vivo*. Anti-FLAG immunoprecipitates from untransfected 293 cells (293) and cells expressing FLAG-tagged β 2-ARs (β 2-AR) or mutant β 2-AR (β 2-AR-EE) were immunoblotted with anti-EBP50. **C**, Disrupting EBP50–ERM interaction increases β 2-AR degradation. Biotin degradation assay of wild-type β 2-ARs in cells expressing native EBP50 (control) or overexpressing truncated EBP50 (EBP50 Δ) or wild-type protein (WT EBP50). Error bars represent s.e. ($n \geq 4$). **D**, The actin cytoskeleton is required for β 2-AR recycling. Latrunculin B treatment inhibited recycling of β 2-ARs (**a–d**) but not of transferrin receptors (TfR; **e–h**).

essential in endocytic sorting of the β 2-AR. To test this idea, we examined endocytic sorting of wild-type β 2-AR in cells pretreated with latrunculin B, which depolymerizes actin¹³. Latrunculin B markedly inhibited recycling of wild-type β 2-ARs and caused internalized receptors to redistribute to perinuclear compartments (Fig. 3Da–d). This manipulation specifically disrupted efficient recycling of β 2-ARs without causing detectable effects on recycling of transferrin receptors in the same cells (indicated by the complete disappearance of labelled transferrin from endocytic structures and dissociation of apo-transferrin)¹⁴ (Fig. 3De–h), even though both receptors recycle by a similar membrane pathway⁷. Efficient recycling of transferrin receptors in the absence of an intact actin cytoskeleton has been shown in previous studies^{13,15}. These results indicate that EBP50, ERM proteins and the actin cytoskeleton have a specialized function in the endocytic sorting of a subset of membrane proteins.

Serine 411 in the –2 position of the PDZ-binding domain (D SLL) of the β 2-AR tail is a potential site for regulatory phosphorylation by G-protein-coupled receptor kinases (GRKs)⁵. GRKs are a family of protein kinases that phosphorylate ligand-activated β 2-ARs and play a key role in rapid desensitization of receptors by promoting receptor–G-protein uncoupling¹⁶ and endocytosis of receptors by clathrin-coated pits¹⁷. Ser411 is phosphorylated *in vitro* by GRK-5 but not GRK-2, even though other residues in the cytoplasmic tail are phosphorylated by both kinases⁵ and both kinases can promote uncoupling and endocytosis of the β 2-AR¹⁸. Although the *in vivo* substrate specificities of GRK-2 and GRK-5 have not been established, precedent from studies of GRK-1-mediated phosphorylation of rhodopsin indicate that kinase specificity may be similar *in vitro* and *in vivo*^{5,19}. As HEK293

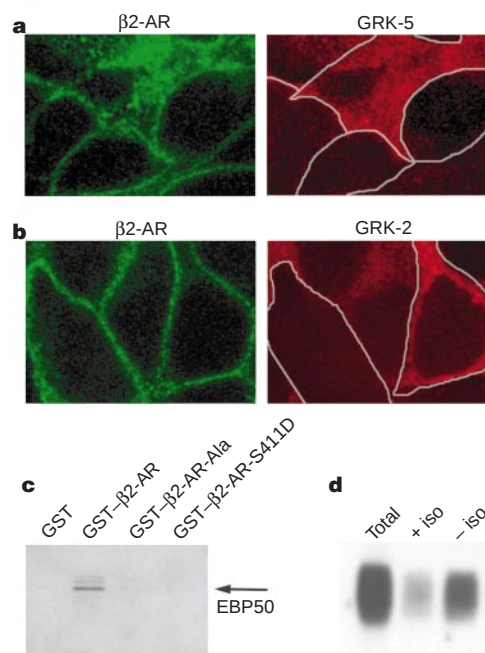


Figure 4 Regulation of β 2-AR sorting by GRK-5. **a**, GRK-5 inhibits recycling of β 2-ARs. Residual antibody-labelled β 2-ARs (green) were observed following agonist removal in endocytic vesicles of transiently transfected cells overexpressing GRK-5 (red) but not adjacent cells not expressing GRK-5. **b**, Efficient recycling of antibody-labelled β 2-ARs (green) was observed in cells overexpressing GRK-2 (red). Lines superimposed on red images indicate cell borders. **c**, S411D mutation disrupts β 2-AR–EBP50 interaction. EBP50 bound specifically to GST fusion proteins containing cytoplasmic tails from wild-type β 2-AR but not β 2-AR-Ala or -S411D. **d**, Agonist-induced degradation of biotinylated β 2-AR-S411D.

cells express relatively high levels of GRK-2 (ref. 18) but not detectable GRK-5 (ref. 20), we examined the effects of overexpressing GRK-5 on β 2-AR trafficking. Recycling of wild-type β 2-AR was inhibited in transfected cells overexpressing GRK-5 but not in adjacent nontransfected cells (Fig. 4a). In contrast, overexpression of GRK-2 had no detectable effect on β 2-AR recycling (Fig. 4b).

Replacing Ser411 with an acidic residue may mimic the effects of protein phosphorylation and disrupts PDZ-mediated protein interaction with NHERF and E3KARP (NHERF-2)¹¹. Replacement of Ser411 with aspartic acid (S411D) blocked interaction of the β 2-AR tail with EBP50 (Fig. 4c) and caused inefficient recycling and ligand-induced degradation of receptors (Fig. 4d). Although acidic substitution of Ser411 does not necessarily mimic the effects of protein phosphorylation, crystallographic and mutational studies indicate that PDZ-mediated protein interactions involve hydrogen-bonding with the free hydroxyl group at the –2 position^{21,22}. Consistent with this, alanine mutation of Ser411 also blocked the interaction of EBP50 with the β 2-AR tail and caused missorting of mutant receptors to lysosomes (not shown). These results indicate that Ser411 is critical for β 2-AR interaction with EBP50 and that GRK-mediated phosphorylation may disrupt this interaction and thereby regulate the sorting of internalized β 2-ARs.

We conclude that sorting of internalized β 2-ARs between recycling and degradative endocytic pathways is controlled by a protein interaction involving the distal carboxyl-terminal cytoplasmic domain of the β 2-AR. We propose that this sorting operation is mediated by PDZ-mediated interaction of the β 2-AR with EBP50/NHERF family proteins. This sorting function also involves inter-

action of EBP50 with ERM proteins and an intact actin cytoskeleton, indicating that EBP50 may link β 2-ARs to the actin cytoskeleton. The efficient recycling of transferrin receptors observed under conditions that inhibit recycling of β 2-ARs indicates that the PDZ-mediated sorting mechanism may specifically control the membrane trafficking of certain signalling receptors, in contrast to the constitutive recycling of many other membrane proteins (including the transferrin receptor) that occurs by 'default' membrane flow²³. Our data provide evidence for a role for PDZ domains and the cortical cytoskeleton in regulating the sorting of receptors after endocytosis. In addition, they suggest a new function for specific GRK isoform(s) in regulating the membrane trafficking of G-protein-coupled receptors after endocytosis. Recycling of internalized β 2-ARs back to the plasma membrane promotes dephosphorylation and functional resensitization of receptors, whereas sorting of internalized receptors to lysosomes promotes downregulation of receptors and long-term desensitization of receptor-mediated signal transduction². Thus, the sorting mechanism identified here may be fundamental in the physiological regulation of signal transduction. □

Methods

Complementary cDNA constructs

N-terminally tagged (haemagglutinin (HA) and FLAG) human β 2-ARs were constructed as described⁷. β 2-AR₁ was constructed by removing the coding sequence for the C-terminal 48 residues. β 2-AR-EE was constructed by appending cDNA encoding the Glu-Glu epitope to the 3' end of the full-length β 2-AR coding sequence. β 2-AR-Ala and S411D were constructed using the Quick-Change mutagenesis kit (Stratagene). Wild-type EBP50 (accession no. AF015926) was C-terminally HA-tagged, and EBP50 Δ was generated by replacing the terminal 61 residues with an HA epitope. All cDNAs were cloned into pcDNA 3.0 (Invitrogen) and verified by dideoxynucleotide sequencing.

Cell culture

HEK293 cells were passaged and stably transfected as described⁷. Cells transiently expressing GRK-2 or GRK-5 (gifts from J. Benovic) were analysed 48 h after transfection.

Degradation of biotinylated receptors

Cells were surface biotinylated²⁴ with 300 μ g ml⁻¹ sulfo-NHS-biotin (Pierce), left at 4 °C to determine total biotinylated proteins, or incubated with or without 10 μ M isoprenaline (RBI) for 4 h. Immunoprecipitation and detection of biotinylated receptors were done as described²⁴. Studies using lysosomal inhibitors (200 μ g ml⁻¹ leupeptin (Sigma), 200 μ M chloroquine (Sigma) or 50 mM NH₄Cl (Fisher Scientific)) were performed by 90 min preincubation before biotinylation, and inhibitors were present during all subsequent manipulations.

Immunofluorescence recycling assay

HA-tagged β 2-ARs were surface labelled with 30 μ g ml⁻¹ HA.11 antibody and cells were incubated with 10 μ M isoprenaline for 15 min to drive agonist-induced internalization. Residual agonist and antibody were removed, and coverslips were immediately fixed (to determine total internalized receptors) or incubated with the antagonist alprenolol (100 μ M; Sigma) for 15, 30 or 60 min at 37 °C before fixation and processing for fluorescence microscopy²⁴. GRK-2 and -5 were detected in permeabilized specimens using 0.8 mg ml⁻¹ rabbit anti-GRK serum (Santa Cruz Biotechnology).

For receptor recycling experiments in the presence of latrunculin B (Alexis Corporation), cells were labelled with HA.11 and 10 μ g ml⁻¹ transferrin Texas Red (to label transferrin receptors). Following endocytosis (induced as above), cells were incubated at 4 °C with 10 μ g ml⁻¹ latrunculin B for 1 h, washed with PBS, warmed to 37 °C and subjected to recycling conditions (as described above) in the presence of 200 μ g ml⁻¹ unlabelled holotransferrin (Sigma).

Recycling of biotinylated receptors

Cells were preincubated with 100 μ g ml⁻¹ leupeptin for 90 min before biotinylation with sulpho-NHS-S-biotin²⁴. Leupeptin was included in all subsequent steps to inhibit lysosomal proteolysis of internalized receptors. Cells were incubated with 10 μ M isoprenaline for 15 min to drive endocytosis of biotinylated receptors, then agonist was washed away and cells were incubated with 100 μ M alprenolol for 0, 5, 15, 30 or 60 min to block additional endocytosis and allow internalized receptors to recycle. Surface-associated biotin was cleaved quantitatively by treatment of intact cells with glutathione and residual biotinylated (internalized) receptors were detected as described²⁴.

Affinity chromatography and western blotting

HEK293 cells were lysed (50 mM Tris, pH 7.4, 1 mM EDTA and protease inhibitors), homogenized and spun at 30,000g for 15 min. The supernatant was solubilized with 1% CHAPS and 150 mM NaCl (final concentration) and re-spun. Extracts were incubated with glutathione S-transferase fusion proteins containing wild-type (last 84 residues) or mutant β 2-AR cytoplasmic tails as described²⁵. Beads were washed with the lysis buffer including 1% CHAPS and 150 mM NaCl. Membranes were probed with a rabbit polyclonal antibody (B61) recognizing EBP50/NHERF/E3KARP⁴ family members. Binding of ³⁵S-methionine-labelled full-length EBP50 to receptor tails was performed as described²⁵.

Co-immunoprecipitation

Cells expressing comparable levels of β 2-ARs were treated with dithiois(succinimidyl-propionate) (DSP) (Pierce), essentially as described²⁶, and FLAG-tagged β 2-ARs were immunoprecipitated with M2-agarose (Sigma). The immunoprecipitates were washed¹² without reducing agents, and bound proteins were extracted in SDS sample buffer with 100 mM DTT and 200 mM β -ME.

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EPS8 and E3B1 transduce signals from Ras to Rac

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The small guanine nucleotide (GTP)-binding protein Rac regulates mitogen-induced cytoskeletal changes and c-Jun amino-terminal kinase (JNK), and its activity is required for Ras-mediated cell transformation¹. Epistatic analysis placed Rac as a key downstream target in Ras signalling²; however, the biochemical mechanism regulating the cross-talk among these small GTP-binding proteins remains to be elucidated. Eps8 (relative molecular mass 97,000) is a substrate of receptors with tyrosine kinase activity³ which binds, through its SH3 domain, to a protein designated E3b1/Abi-1 (refs 4, 5). Here we show that Eps8 and E3b1/Abi-1 participate in the transduction of signals from Ras to Rac, by regulating Rac-specific guanine nucleotide exchange factor (GEF) activities. We also show that Eps8, E3b1 and Sos-1 form a tri-complex *in vivo* that exhibits Rac-specific GEF activity *in vitro*. We propose a model in which Eps8 mediates the transfer of signals between Ras and Rac, by forming a complex with E3b1 and Sos-1.

Rac proteins belong to the sub-family of Rho-GTPases that become activated when bound GDP is exchanged for GTP. Within this family, Rho regulates the assembly of stress fibres, Rac controls membrane ruffling and lamellipodia and Cdc42 mediates the formation of filopodia. Rho-GTPases are organized in hierarchical cascades, in which activated Ras or Cdc42 can independently activate Rac^{1,2}. Phosphatidylinositol 3-phosphate kinase (PI(3)K) is the immediate downstream effector of Ras⁶, and might activate Rac through the products of its catalytic activity, phosphoinositide phosphates (PtdInsPs), which can bind and modulate the activity of GEFs for Rac, like Vav and h-Sos-1 (refs 7, 8). Initial results indicated that overexpression of Eps8 increased JNK activity, leaving mitogen-activated protein kinase (MAPK) unperturbed, leading to the testable hypothesis that Eps8 might be involved in the regulation of Rac (G.S. and P.P.D.F., unpublished observations).

We engineered an *eps8* targeting vector (Fig. 1a) and used a

targeted embryonic stem-cell clone (Fig. 1b) to derive mice heterozygous and homozygous for the *eps8* mutated allele. Eps8-null mice displayed no obvious phenotype. We established *Eps8* $+/+$, $+/-$ and $-/-$ immortalized fibroblast-like cell lines that displayed the predicted expression of Eps8 (Fig. 1c) and comparable levels of the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) (see Supplementary Information). PDGF induced actin reorganization and membrane ruffles in $+/+$ but not in $-/-$ (Fig. 1d). Re-expression of Eps8 in $-/-$ cells restored ruffle formation (Fig. 1d). Similar results were obtained with EGF (data not shown). 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which induces actin reorganization through mechanisms that are different from those of growth factors⁹, induced ruffling in both $-/-$ and $+/+$ fibroblasts. Thus Eps8 acts specifically on growth-factor-dependent actin remodelling.

Expression vectors encoding the activated versions of Ras, Rac, Cdc42 and PI(3)K (RasV12, RacQL, Cdc42QL and rCD2p110, respectively) induced ruffling in $+/+$ cells (Fig. 2). In $-/-$ cells, RasV12 and CD2p110 did not induce ruffles (Fig. 2), despite readily detectable synthesis and intrinsic activity of both proteins (see Supplementary Information). Microinjection of vectors for

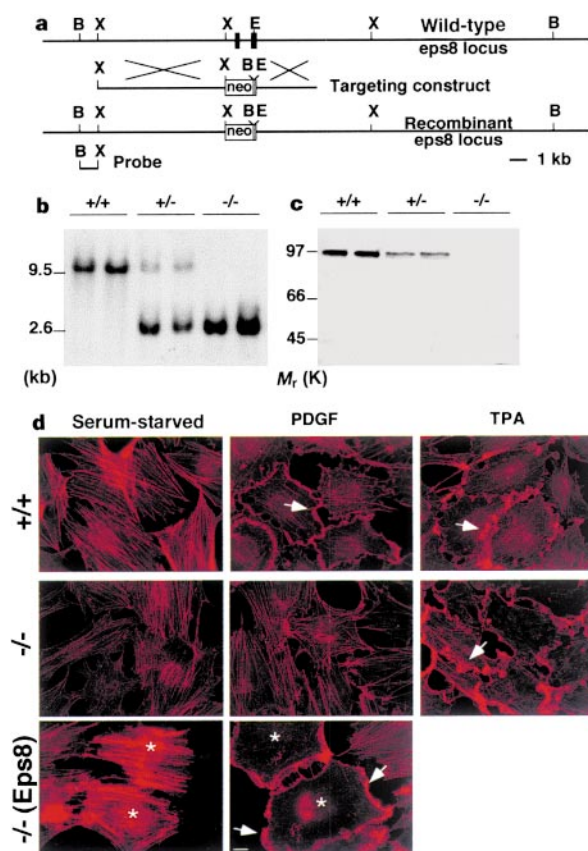


Figure 1 Disruption of *eps8* locus abrogates PDGF-induced membrane ruffling.

a, Restriction map of the mouse *eps8* locus and of the targeting construct. B, *Bam*HI; X, *Xba*I; E, *Eco*RV; solid bars indicate the two exons encoding the SH3 domain. **b**, Southern blot of *Bam*HI-digested mouse tail DNAs from $2+/+$, $2+/-$ and $2-/-$ mice, probed with the 1-kb fragment indicated in **a**. **c**, Immunoblot, with the anti-Eps8 peptide serum (amino acids 32–55) Eps8 antibody, of cellular lysates from *eps8* $+/+$, $+/-$ and $-/-$ fibroblasts (four independently derived clones in each case, of which two are shown). **d**, *Eps8* $+/+$ (top) and $-/-$ (middle and bottom) fibroblasts were treated as indicated and stained for actin. In the bottom panels $-/-$ cells were also microinjected with pCR3-*eps8* ($-/-$ (Eps8)). Results in this and all subsequent experiments are representative of independently derived clones of $+/+$ and $-/-$ fibroblasts (4 of each). Asterisks identify microinjected cells; arrowheads point to ruffles. Bar represents 10 μ m.

RasV12 or rCD2p110 together with Eps8 restored ruffling in $-/-$ cells (Fig. 2). Moreover, a dominant-negative Ras inhibited PDGF-induced ruffles, indicating that actin reorganization induced by receptors with tyrosine kinase activity (RTKs) may be Ras-dependent in our mouse embryo fibroblasts (data not shown). Thus, the genetic localization of Eps8 is between PI(3)K and Rac.

To obtain direct biochemical evidence for a role of Eps8 as an upstream regulator of Rac, we used the Cdc42/Rac Interactive Binding region (CRIB) of α Pak, which binds specifically to activated GTP-bound Rac *in vivo*¹⁰. Sepharose-immobilized glutathione *S*-transferase-labelled CRIB (GST-CRIB) bound significantly better ($1.7\text{-fold} \pm 0.2\text{ s.e.}$) to GTP-bound Rac in lysates of PDGF-treated $+/+$ cells, compared with $-/-$ cells (Fig. 3a). Thus, Eps8 is directly involved in the activation of Rac *in vivo*. Eps8 might activate Rac by inhibiting a specific GTPase-activating protein (GAP) or activating a GEF. In cellular lysates from $+/+$ or $-/-$ fibroblasts, we could not detect differences in GAP activities toward Rac (see Supplementary Information). We then measured GEF activity using an assay that can effectively detect this activity, as demonstrated by increased Rac-GEF in cellular lysates of Cos-7 cells transfected with the DH (Dbl homology) domain of Sos-1 (Fig. 3b; ref. 8). In lysates from $-/-$ cells, Rac-

GEF, but not Cdc42-GEF, activity was threefold lower than in $+/+$ cells, an effect reverted by re-expression of Eps8 in $-/-$ cells ($-/-$ (Eps8) cells; Fig. 3c).

Sos-1 has been implicated in the transmission of signals from Ras/PI(3)K to Rac⁸. Immunodepletion of Sos-1 from $+/+$ or $-/-$ (Eps8), but not $-/-$, lysates resulted in a substantial decrease in activity of Rac-GEF (Fig. 3d) but not of Cdc42-GEF (data not shown). Immunodepletion of Eps8 from $+/+$ or $-/-$ (Eps8) lysates caused a marked reduction in GEF activity, which become comparable to that detected in $-/-$ lysates or in Sos-1-depleted $+/+$ lysates (Fig. 3d). Thus Eps8 is directly responsible for activation of a Rac-specific GEF activity, which is probably represented by Sos-1. E3b1 (also known as Abi-1) and RN-tre interact with the SH3 domain of Eps8 (refs 4, 5, 11). Immunodepletion of E3b1, but not of RN-tre, from $+/+$ lysates led to a substantial reduction in Rac-GEF activity (Fig. 3d). Co-immunodepletion of Eps8 and E3b1 did not produce additive effects, indicating that they are required simultaneously (Fig. 3d).

The above results led us to test whether E3b1 and/or Eps8 could physically interact with Sos-1. Native Sos-1 could be recovered with substantial stoichiometry onto GST-E3b1 and GST-E2 (amino acids 331–480 of E3b1), but not onto GST or GST-E1 (amino acids 1–330 of E3b1). GST-E3b1 and GST-E2 also interacted with a

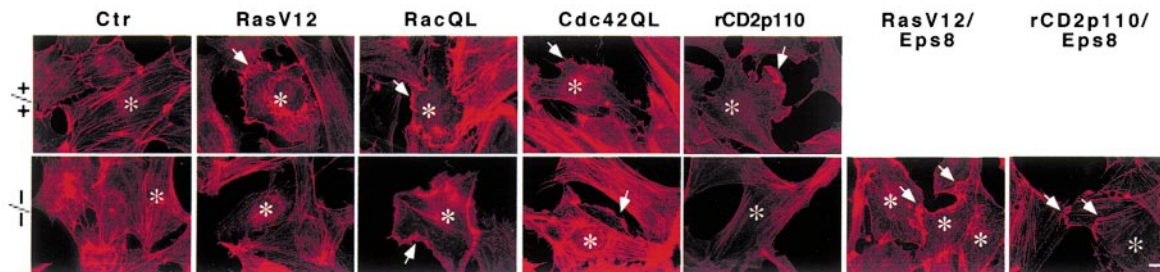


Figure 2 Epistatic localization of Eps8 in the Ras pathway. Fibroblasts ($+/+$, top and $-/-$, bottom) were microinjected with the indicated plasmids and stained for actin. Asterisks identify microinjected cells and arrows point to ruffles. Bar represents 10 μm .

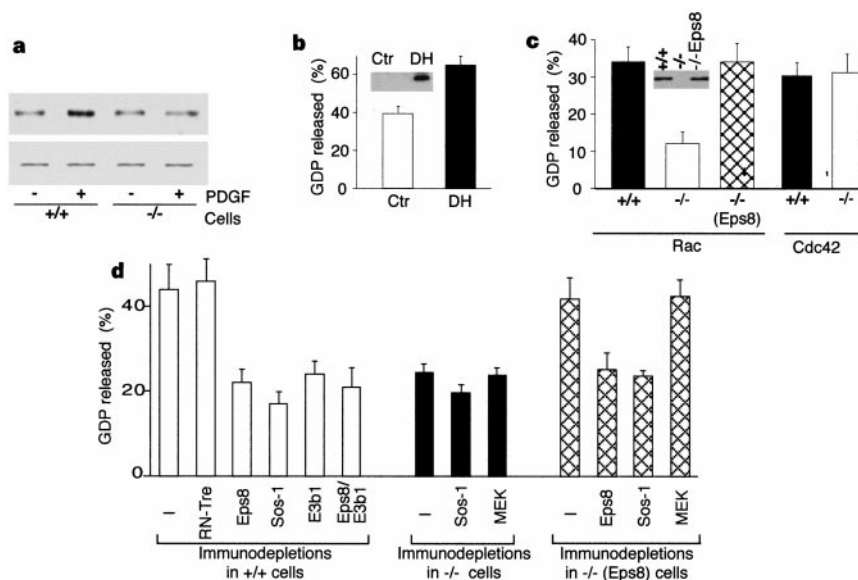


Figure 3 Eps8 activates Rac and a Rac-specific GEF. **a**, $+/+$ and $-/-$ fibroblasts were treated with PDGF (+) or mock-treated (-). Lysates were incubated with GST-CRIB and detected with anti-Rac (top), or directly immunoblotted with anti-Rac (bottom). **b**, GEF activity in lysates from Cos-7 cells transfected with an empty vector (Ctr) or with the HA-epitope-tagged DH domain of Sos-1 (DH). Inset, DH expression. **c**, GEF activity towards

Rac or Cdc42 in lysates from $+/+$, $-/-$ or $-/-$ (Eps8) cells. Inset, Eps8 expression. **d**, GEF activity in lysates from $+/+$, $-/-$ or $-/-$ (Eps8) cells immunodepleted of the indicated proteins. In all cases, immunodepletion removed $>95\%$ of the indicated proteins (see Supplementary Information).

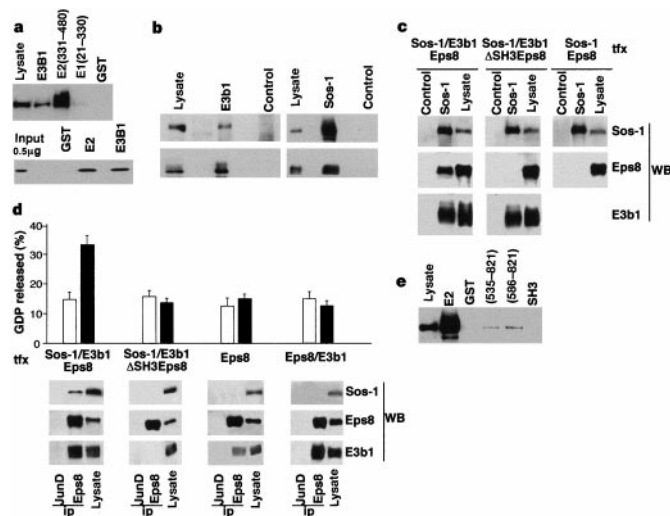


Figure 4 Eps8, E3b1 and Sos-1 form a tri-complex with Rac-GEF activity. **a**, Binding of GST fusion proteins to Sos-1 present in lysates of Sos-1-transfected Cos-7 cells (top, detection with anti-Sos-1) or to Sos-1-His (bottom, detection with anti-histidine). **b**, Lysates from Cos-7 cells were immunoprecipitated with the antibody indicated on the top (control refers to the corresponding pre-immune serum), followed by detection with anti-Sos-1 (top) or anti-E3b1 (bottom). **c**, Lysates, from Cos-7 cells transfected with the indicated cDNAs (tfx) were immunoprecipitated with anti-Sos-1 or pre-immune sera (control), followed by detection (western blot, WB) with anti-Sos-1 (top), anti-Eps8 (middle) or anti-E3b1 (bottom) antibodies. **d**, Rac-GEF activity (top) in immunoprecipitates (ip) performed with anti-Eps8 (closed bars) or an irrelevant antibody (anti-JunD, empty bars) on lysates of Cos-7 cells transfected with the indicated plasmids (tfx). The same immunoprecipitates were analysed by immunoblotting (WB) with the indicated antibodies (bottom panels). **e**, Binding of GST fusion proteins to Sos-1 present in lysates of Sos-1-transfected Cos-7 cells. Lanes labelled 'lysate' were loaded with 50 µg of the appropriate lysate.

purified, histidine-tagged carboxy-terminal region (Sos-1-His, amino acids 1131–1333) of Sos-1 (ref. 12), indicating that the binding is direct (Fig. 4a). Finally, Sos-1 could be co-immunoprecipitated with E3b1 and vice versa (Fig. 4b).

The interaction between E3b1 and Sos-1, and that previously shown between Eps8 and E3b1 (ref. 4), indicated that these three proteins may form a tri-complex in which E3b1 holds together Eps8 and Sos-1. Eps8 and Sos-1 should then be co-immunoprecipitable in an E3b1-dependent manner. We could not show co-immunoprecipitation between endogenous Eps8 and Sos-1 (not shown). However, when we co-transfected expression plasmids encoding for Eps8, E3b1 and Sos-1, we could readily recover Eps8 (and E3b1) in Sos-1 immunoprecipitates (Fig. 4c) and vice versa (Fig. 4d). An Eps8 mutant¹³ with a 5-amino-acid deletion in its SH3 domain, ΔSH3Eps8, cannot interact with E3b1 (Fig. 4d) and is unable to complement the genetic defect of Eps8^{−/−} cells (data not shown). In ΔSH3Eps8/E3b1/Sos-1 transfectants, there was no co-immunoprecipitation between mutant Eps8 and Sos-1 (Fig. 4c, d). Finally, if Eps8 and Sos-1 were overexpressed in the absence of E3b1, there was no co-immunoprecipitation between the two proteins (Fig. 4c).

A tri-complex can thus be formed *in vivo* in which E3b1 holds together Eps8 and Sos-1. In this tri-complex, Eps8 might physically interact with Sos-1. Indeed, we showed that native Sos-1 can bind, albeit with low stoichiometry probably reflecting low affinity, to immobilized GST-Eps8(536–821) and GST-Eps8(586–821), encompassing the last 285 and 235 amino acids of Eps8, respectively (Fig. 4e). GST-Eps8(586–821) does not contain the SH3 domain, so the interaction cannot have been mediated by Sos-1-bound E3b1.

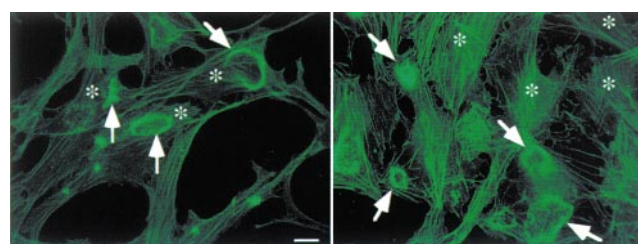


Figure 5 Inhibition of ruffling by anti-E3b1 antibodies. NIH-3T3 fibroblasts were microinjected with rabbit IgG (left panel), or affinity-purified rabbit polyclonal antibodies against E3b1 (right panel), followed by treatment with PDGF and staining for actin. Arrows point to dorsal ruffles. Asterisks identify microinjected cells. Bar represents 10 µm.

Thus, while Eps8 and Sos-1 cannot interact *in vivo* in the absence of E3b1, they possess surfaces capable of interacting with each other, at least *in vitro*.

We performed GEF assays on Eps8 immunoprecipitates, under conditions that allow the detection of the tri-complex *in vivo* (Fig. 4d). There was substantially more Rac-GEF activity in Eps8 immunoprecipitates from Eps8/E3b1/Sos-1 transfectants than in control immunoprecipitates or immunoprecipitates from ΔSH3Eps8/E3b1/Sos-1, Eps8/E3b1 or Eps8-alone transfectants (Fig. 4d), thus supporting the proposal that Sos-1 and E3b1 are required for Eps8-dependent activation of Rac.

The requirement for E3b1 in Rac activation was further validated by microinjecting NIH/3T3 cells with anti-E3b1 antibodies, which led to abrogation of ruffling upon PDGF stimulation (Fig. 5), leaving TPA-induced ruffling unperturbed (data not shown), a phenotype indistinguishable from that of Eps8^{−/−} cells.

By positioning Eps8 in the mitogenic network, we showed that it participates in the transduction of signals from Ras/PI(3)K to Rac. The genetic positioning of Eps8 downstream of PI(3)K is compatible with a biochemical action either linearly downstream of PI(3)K or in a parallel, necessary but not sufficient, pathway. Thus PI(3)K, probably through PtdInsPs, and Eps8 might be simultaneously required for the activation of Rac; alternatively Eps8 might be directly regulated by PI(3)K/PIPs. Eps8 participates in the activation of Rac by regulating Rac-GEF activity(ies). Biochemical evidence identified Sos-1 as the GEF controlled by Eps8, although we cannot formally exclude the involvement of other GEFs. Eps8 and Sos-1 participate *in vivo* in a tri-complex, endowed with Rac-GEF activity *in vitro*, in which E3b1 serves as a scaffolding protein. The physical requirement for both Eps8 and E3b1 is supported by biochemical and biological evidence presented here. In this tri-complex, Eps8 might directly contact Sos-1, as supported by our demonstration that Eps8 binds to Sos-1 *in vitro*, with low stoichiometry, possibly reflecting low affinity. Thus, in the simplest scenario, the quaternary structure of the complex might be responsible for the activation of the Rac-GEF activity of Sos-1.

Cytoskeletal rearrangements, in response to extracellular stimuli, are linked to fundamental cellular processes^{1,2}. However, the Eps8 knock-out mice are viable and fertile and, so far, devoid of any macroscopic abnormality (see Supplementary Information). One possible explanation is that the growth-factor-induced remodelling of cytoskeleton is not an essential step in RTK-mediated signalling. Conflicting data have been reported in this regard^{14–16}. Alternatively, pathways involving Eps8 could be bypassed as a consequence of their redundancy *in vivo*, as indirectly suggested by the existence of a family of Eps8-related genes identified in the EST data base (not shown). The function(s) of these genes is unknown. However, based on the role of Eps8 in mediating the exchange of signals between Ras and Rac, the hypothesis that Eps8-like molecules

might represent a universal link between small G proteins of the Rho-family deserves further investigation. □

Methods

Expression vectors and antibodies

The expression vectors used were pCR-myc-eps8, pCR3- Δ SH3Eps8 (ref. 13), pDCR-H-RasV12 (ref. 14), pCD2p110 (ref. 17), pSG5-HA-PKB¹⁶, pMT2-Sos-1 (ref. 18), pCEFL-HA-E3b1 (ref. 4), pCDNA3RacQ61L (RacQL) and pCDNA3CDC42Q61L (Cdc42QL)¹⁹. Corresponding empty vectors were used as controls when appropriate (Cfr in Figures). Antibodies (Ab) used were: anti-eps8 and anti-e3b1 sera^{3,4}; an anti-Eps8 (amino-acid positions 32–56 of the murine Eps8 protein) peptide serum; anti-ERK1 and anti-Sos-1 polyclonal sera (Santa Cruz Biotechnology); a rat monoclonal anti-v-H-Ras (AB-2, Oncogene Science); and a mouse monoclonal anti-Rac-1 (Transduction Laboratory).

Generation of eps8-null mice and fibroblasts

Mouse genomic eps8 clones were isolated from a 129SV library (Stratagene). A 7-kilobase (kb) *XhoI*–*XhoI* fragment was used for 5' homology and a 2.5-kb *EcoRV*–*NotI* fragment for 3' homology. A PGK–neo cassette replaced an exon-containing eps8 genomic 1.7-kb *XhoI*–*EcoRV* fragment. The eps8 SH3 domain is encoded by 2 exons (Fig. 1a) and the targeting construct excluded the first and part of the second of these exons. A genomic probe, flanking the targeting construct at the 5' end, was used to detect the wild-type (2.6 kb) and the targeted (9.5 kb) alleles (Fig. 1a). Electroporation into mouse E14 ES cell clones, and subsequent manipulations leading to mice heterozygous and homozygous for the mutant eps8 allele, were as described²⁰. Immortalized fibroblasts from +/+, +/- or -/- embryos were established as described²⁰ (see also Supplementary Information).

Microinjection, immunofluorescence and biochemical assays

Fibroblasts were seeded on gelatine-coated glass coverslips and serum-starved for 24 h. Expression vectors (0.1 mg ml⁻¹) were microinjected in the cell nuclei. Successful injections were assessed either by direct staining of the protein encoded by the injected complementary DNA or by detection of a co-injected GFP expression plasmid. At least 100 microinjected cells were analysed for each experiment. Three hours after injection (4 h in the case of Cdc42QL), cells were treated with 10 ng ml⁻¹ of PDGF or 100 ng ml⁻¹ of EGF (Upstate Biotechnology) or mock treated for 10 min, fixed and stained with TRITC-labelled phalloidin to detect actin filaments, using standard procedures⁹.

GEF and CRIB assays were performed as described^{21,22}. All presented data are the mean $\pm$ s.e. of at least three independent experiments. In GEF assays, results are expressed as a percentage of the [³H]GDP released after 20 min relative to time 0, after subtracting the background counts released in control reaction (buffer alone).

In vitro bindings, immunoprecipitation and co-immunoprecipitation were performed as described³.

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Supplementary Information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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A *Salmonella* protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion

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An essential feature of the bacterial pathogen *Salmonella* spp. is its ability to enter cells that are normally non-phagocytic, such as those of the intestinal epithelium¹. The bacterium achieves entry by delivering effector proteins into the host-cell cytosol by means of a specialized protein-secretion system (termed type III), which causes reorganization of the cell's actin cytoskeleton and ruffling of its membrane^{2–4}. One of the bacterial effectors that stimulates these cellular responses is SopE, which acts as a guanyl-nucleotide-exchange factor on Rho GTPase proteins such as Cdc42 and Rac (ref. 5). As the actin-cytoskeleton reorganization induced by *Salmonella* is reversible and short-lived, infected cells regain their normal architecture after bacterial internalization^{6,7}. We show here that the *S. Typhimurium* effector protein SptP, which is delivered to the host-cell cytosol by the type-III secretion system, is directly responsible for the reversal of the actin cytoskeletal changes induced by the bacterium. SptP exerts this function by acting as a GTPase-activating protein (GAP) for Rac-1 and Cdc42.

We identified a *S. Typhimurium* mutant that can induce actin-cytoskeleton rearrangements and membrane ruffling in host cells but is unable to reverse these effects after bacterial internalization (Fig. 1). This *S. Typhimurium* mutant strain carries a null mutation in *sptP*, a gene that encodes an effector protein for delivery to the host cell by the invasion-associated type-III secretion system^{8,9}. Ref52 cells infected with wild-type *S. Typhimurium* or the complemented mutant strain began to regain a normal appearance of their

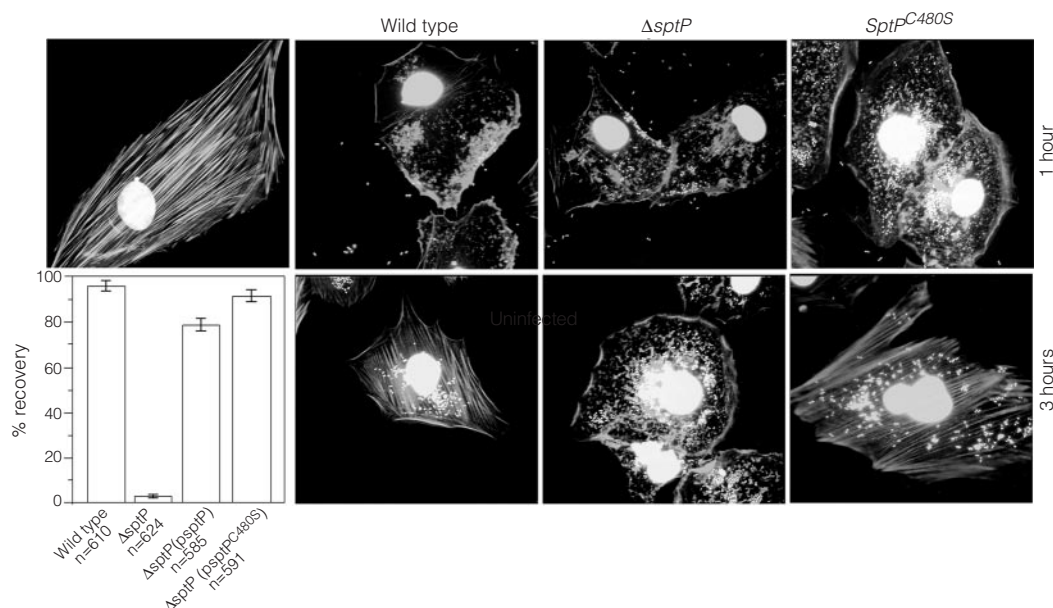


Figure 1 SptP mediates the recovery of the normal organization of the actin cytoskeleton after *S. Typhimurium* internalization. Ref52 cells were infected with different strains of *S. Typhimurium* and the actin cytoskeleton visualized by rhodamine-phalloidin staining at 1 h and 3 h after infection. Bacteria were visualized by DAPI staining. Phalloidin and DAPI images were individually captured with a Hamamatsu 75i CCD camera and merged using

Adobe Photoshop. The percentage of cells that recovered their actin-cytoskeleton architecture 3 h after infection with different bacterial strains is shown. Values are the mean and standard deviations from three independent experiments with wild-type, $\Delta sptP$, or the mutant strain expressing either wild-type SptP (*psptP*) or mutant SptP(C480S) (*psptP^{C480S}*) from integrated plasmids.

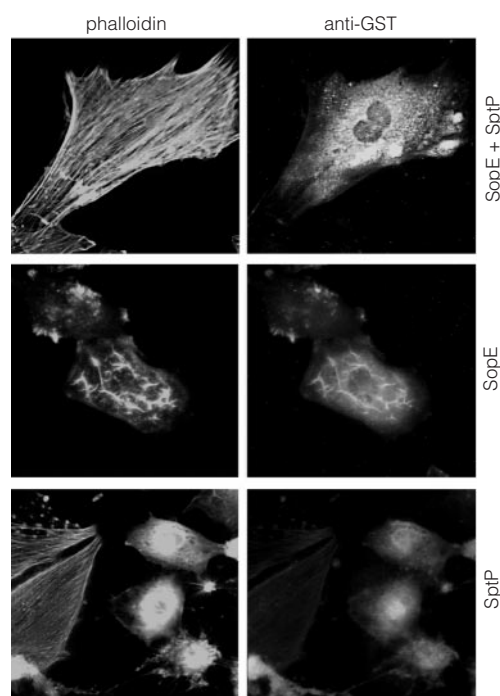


Figure 2 SptP and SopE antagonize each other's function. Ref52 cells were microinjected with GST-SopE or GST-SptP, or with a mixture of both. The concentration of bacterial protein was 400 $\mu\text{g ml}^{-1}$. When administered individually, equivalent amounts of purified GST were added to the microinjection solution to maintain a constant total protein concentration. The actin cytoskeleton was visualized by rhodamine-phalloidin staining 45 min after microinjection. Microinjected cells were identified by staining with an antibody against GST (Sigma). In each case, at least 100 microinjected cells were examined in 3 independent experiments and gave the same results as those shown here.

actin cytoskeleton as soon as 80 min after infection, and by 3 h most of the cells had fully recovered, despite the large number of internalized bacteria (Fig. 1, and data not shown). In contrast, cells infected with the *sptP* mutant strain continued to ruffle 3 h after infection and so did not regain a normal actin cytoskeletal architecture (Fig. 1, and data not shown). These results indicate that *S. typhimurium* is active in the process that leads to the rebuilding of the actin cytoskeleton after bacterial infection, presumably through the function of the type-III secreted protein SptP.

SptP is a modular protein consisting of two discrete domains⁸. The amino-terminal region shares sequence similarity with ExoS of *Pseudomonas aeruginosa*¹⁰ and YopE of *Yersinia* spp.^{11,12}. The carboxy-terminal domain of SptP shares sequence similarity with the *Yersinia* YopH protein and other tyrosine phosphatases. Purified SptP disrupts the actin cytoskeleton when microinjected into host cells, although it does so in a tyrosine-phosphatase-independent manner^{8,9}. ExoS, YopE and YopH also disrupt the actin cytoskeleton and are delivered into host cells by related type-III secretion systems encoded by these bacteria^{13–16}.

We investigated whether the tyrosine phosphatase activity of SptP was necessary for rebuilding the actin cytoskeleton after *S. Typhimurium* infection by constructing a *S. Typhimurium* strain that expresses a mutant form of SptP (SptP(C480S)) with a critical cysteine residue at its catalytic site substituted by serine, which therefore has no tyrosine phosphatase activity⁹. Ref52 cells infected with this mutant strain rebuilt their actin cytoskeleton in a way that was indistinguishable from that observed after infection with wild-type *S. Typhimurium* (Fig. 1). Results were similar with strains expressing a truncated form of SptP lacking its last 60 amino acids which was therefore also devoid of tyrosine phosphatase activity (data not shown). These results indicate that the SptP-mediated rebuilding of the actin cytoskeleton is independent of its tyrosine phosphatase activity.

We have previously shown that microinjection of purified *S. Typhimurium* SopE protein stimulates extensive membrane ruffling and actin cytoskeleton reorganization, consistent with its role as

an exchange factor for Rho GTPases⁵. To investigate the effect of SptP on SopE-mediated actin cytoskeletal rearrangement, we microinjected purified SopE and SptP together and examined the architecture of the actin cytoskeleton by rhodamine-phalloidin staining. Simultaneous microinjection of SptP and SopE prevented the membrane ruffling and actin cytoskeleton rearrangement normally stimulated by microinjection of SopE alone⁵ (Fig. 2) and blocked disruption of the actin cytoskeleton that is seen after microinjection of SptP alone⁹ (Fig. 2). These results indicate that when simultaneously applied to cells, SptP and SopE can antagonize each other's effect on the actin cytoskeleton.

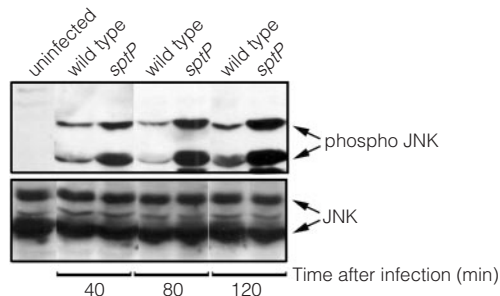


Figure 3 Effect of SptP on *S. Typhimurium*-mediated JNK activation. Ref52 cells were infected for various times with either wild-type *S. Typhimurium* or a *sptP*-deletion mutant at a multiplicity of infection of 40. The levels of phosphorylated (active) JNK were determined by western-blot analysis and densitometric scanning (see Methods; upper panel). The blot was stripped and reprobed with an antibody directed against JNK (Santa Cruz Biotechnology) (lower panel).

As well as causing actin cytoskeleton rearrangement, *S. Typhimurium* infection results in the activation of the MAP-kinase pathways involving p38 protein and JNK kinase, which trigger a nuclear response and cause proinflammatory cytokines to be produced¹⁷. These responses depend on Cdc42 and Rac-1 and are mediated by bacterial effectors delivered by the type-III secretion system, such as that of SopE (refs 2, 3, 5). We investigated whether SptP could also antagonize bacterial signals leading to the activation of SAPK/JNK kinases. Ref52 cells were infected for various times with either wild-type *S. Typhimurium* or the *sptP* isogenic mutant and the activation of SAPK/JNK kinase was tested. As shown in Fig. 3, SAPK/JNK activation was lower in cells infected with wild-type *S. Typhimurium* than in cells infected with the *sptP* mutant. Activation of SAPK/JNK peaked 30 to 40 min after infection with wild-type *Salmonella* and declined slightly over time (Fig. 3, and data not shown). In contrast, the amount of the phosphorylated (active) form of SAPK/JNK present was significantly higher (3–5-fold in three independent experiments) in cells infected with the *sptP* mutant, and remained high even 120 min after infection (Fig. 3). These results indicate that SptP downregulates the SAPK/JNK activation stimulated by *S. typhimurium* infection.

Rho GTPases exert a variety of effects through different downstream proteins (reviewed in refs 18, 19), and the activation of the SAPK/JNK pathway and the actin cytoskeleton rearrangements mediated by the Rho GTPases Cdc42 and Rac-1 are thought to be mediated by different effector proteins. The results indicating that SptP can antagonize signals leading to both cytoskeletal and nuclear responses stimulated by either *S. Typhimurium* infection or microinjection of SopE suggest that SptP may act directly on the Rho GTPases. We therefore investigated whether SptP could interact with Rac-1 by using a glutathione-S-transferase (GST)

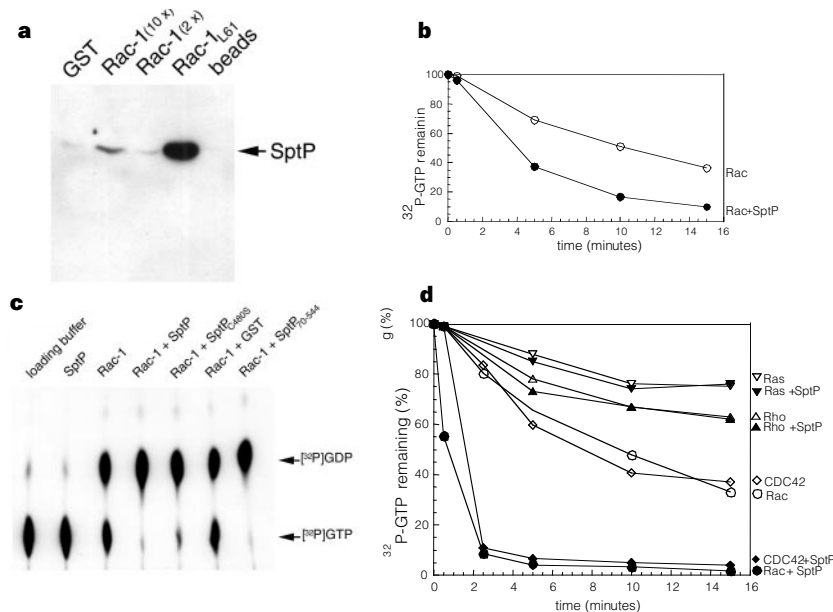


Figure 4 SptP is a GAP for CDC42 and Rac. **a**, SptP binds Rac(L61). Bacterial extracts containing SptP were incubated with glutathione-agarose beads loaded with GST, GST-Rac and GST-Rac(L61), and bound proteins were examined by western blot using a monoclonal antibody against SptP. The amount of GST-Rac protein used in the assay was 2 (2x) and 10 (10x) fold more than GST-Rac(L61). Equivalent results were obtained when purified SptP was used in the assay (see Methods). **b**, **c**, SptP stimulates the GTPase activity of Rac. The effect of addition of purified GST-SptP(70-544) (10 nM) to purified [γ -³²P]GTP-loaded GST-Rac-1 (400 nM) was examined in a filter-binding GAP assay (see Methods) (**b**) (values represent results from one of four experiments that gave equivalent results, with a standard deviation of less than 5%). Alternatively, the GAP activity of GST-

SptP (100 nM) towards purified [α -³²P]GTP-loaded GST-Rac-1 (400 nM) was examined by TLC (**c**) (this experiment was repeated three times with identical results). **d**, GAP activity of SptP shows preference for Rac and Cdc42. The GAP activity of GST-SptP(70-544) towards Rac-1, Cdc42Hs, RhoA and H-Ras was examined by a filter-binding assay (see Methods). The concentration of GST-SptP(70-544) was 40 nM for reactions with Cdc42 and Rac, 160 nM for reactions with Rho, and 400 nM for reactions with Ras. The concentration of all small G proteins in the reactions was 400 nM (values represent the results of one of three experiments that gave equivalent results, with a standard deviation of less than 5%).

pull-down assay. Wild-type Rac-1 and its constitutively active mutant form Rac-1(L61) were purified as GST-fusion proteins and tested for their ability to interact with wild-type SptP expressed in *Escherichia coli*. SptP was able to interact with GST-Rac-1(L61) but not with either GST-Rac-1 or GST alone (Fig. 4a), indicating that SptP can bind Rac-1 in an interaction that is preferentially or exclusively directed to the GTP-bound form of this GTPase.

The function of small GTP-binding proteins is negatively regulated by a family of GTPase-activating proteins (GAPs) which preferentially bind the active (GTP-bound) form of the cognate G protein and stimulate its intrinsic GTPase activity^{18–20}. The observations that SptP antagonizes bacterial responses resulting from the activation of the Rho GTPases Cdc42 and Rac-1 and that SptP preferentially or exclusively binds the GTP-bound form of Rac-1 suggested that SptP might function as a GAP to downregulate signalling through Rho GTPases. We therefore tested the ability of SptP to stimulate the intrinsic GTPase activity of Rac-1 and Cdc42 and found that SptP had potent GAP activity for Rac-1 (Fig. 4b, c) and Cdc42 (Fig. 4d). The SptP GAP activity was readily detected

even at 40-fold molar excess of Rac-1 over SptP (Fig. 4b) and showed a slight preference for Rac-1 over Cdc42 (Fig. 4d, and data not shown). In contrast, SptP had no measurable GAP activity towards Ras and its GAP activity towards Rho was 100-fold less than towards Rac (Fig. 4d, and data not shown), indicating specificity for a subset of Rho GTPases. SptP(C480S), which has no tyrosine phosphatase activity, retained wild-type GAP activity (Fig. 4c), consistent with our observation that tyrosine phosphatase activity is not required for modulating effects of SptP on the actin cytoskeleton.

Proteins with GAP activity towards Rho GTPases share a sequence motif containing an invariant arginine that is essential for efficient catalysis²⁰. Inspection of the amino-acid sequence of SptP, as well as of the related bacterial proteins ExoS and YopE, revealed the presence of a similar sequence motif (Fig. 5a). We therefore tested whether this motif was involved in the catalytic activity of SptP by constructing an SptP mutant in which the invariant arginine was replaced by alanine. The resulting protein (SptP(R209A)) could still bind Rac(L61) (data not shown), indicating that the mutation did not cause a gross conformational change. As shown in Fig. 5b, SptP(R209A) had no GAP activity towards Rac, indicating that SptP works by a mechanism similar to that used by other GAPs²⁰. To determine whether this GAP activity of SptP mediated the rebuilding of the actin cytoskeleton following infection with wild-type *Salmonella*, we constructed a *S. Typhimurium* strain expressing SptP(R209A). This strain expressed and secreted the mutant protein at wild-type levels (data not shown). Cells infected with the *S. Typhimurium* strain expressing SptP(R209A) failed to regain the normal appearance of the actin cytoskeleton, confirming the importance of SptP GAP activity in this process (Fig. 5c).

We have shown that the *S. Typhimurium* type-III secreted protein SptP modulates the actin cytoskeleton by acting as a GAP for Rac-1 and Cdc42. A similar mechanism may account for the activity of the related toxins ExoS and YopE which also disrupt the actin cytoskeleton. The GAP activity of SptP results in down-regulation of the actin-cytoskeleton rearrangements stimulated by other bacterial effectors delivered by the type-III secretion system, such as the Rho-GTPase exchange factor SopE. Our results provide a molecular explanation for the rapid reversibility of cellular responses associated with *S. Typhimurium* entry into cells. Also relevant may be the selectivity of SptP for Rac-1 and Cdc42, and its lack of GAP activity towards Rho. As SopE activates Rho, at least *in vitro*⁵, such activity may help to rebuild the actin cytoskeleton after bacterial internalization, given that activation of Rho leads to the assembly of stress fibres and focal adhesions^{18,19}. The downregulation of cellular responses may help to maintain the viability of cells that provide a permissive environment for the bacteria to replicate or evade host defences, because prolonged signalling through Cdc42 and Rac mediated by other bacterial effectors may harm the cell. Our results also indicate that *Salmonella* delivers SopE and SptP into the host cell either sequentially or in different amounts in order to stimulate the responses associated with the infectious process. The coordinated regulation of Rho GTPases by bacterial effector proteins that can sequentially step up and reduce their activities is a remarkable example of a strategy evolved by a pathogen to manipulate the cellular functions of its host. □

Methods

Bacterial strains and plasmids

Wild-type *S. Typhimurium* strain SB300 has been described⁹. The isogenic strains SB749, which carry a complete deletion of the *sptP* gene, SB1001 and SB1002 which express wild-type SptP or the catalytic mutant SptP^{C480S}, respectively, from integrated plasmids were constructed by site-directed mutagenesis and allelic exchange. Plasmids expressing fusion proteins between glutathione-S-transferase (GST) and wild-type or mutant forms of SptP have been described⁹. Plasmids expressing fusion proteins between GST and different Rho GTPases were from different sources and have also been described^{2,5}. SptP^{R209A} was

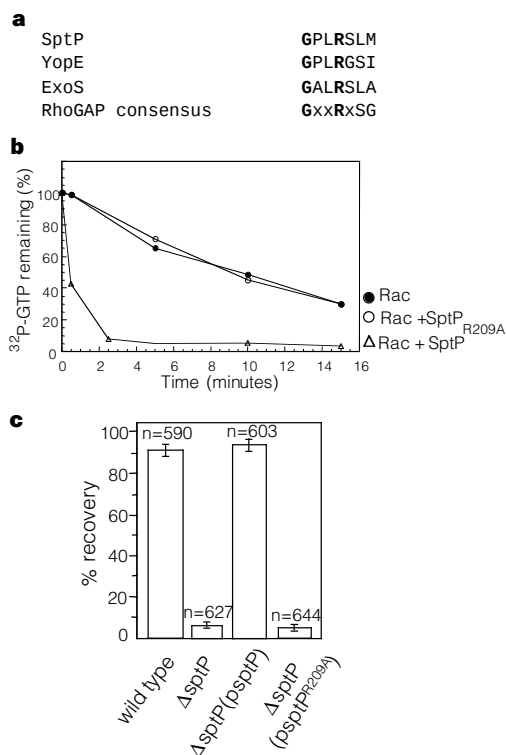


Figure 5 An 'arginine finger' characteristic of Rho-GTPase-activating proteins is required for SptP function. **a**, SptP displays a conserved motif (arginine finger) present in RhoGAPs and related bacterial toxins. **b**, SptP(R209A), which carries a mutation in the arginine finger motif, is devoid of GAP activity. The GAP activity of GST-SptP(70–544, R209A) (1 μM) towards [γ-³²P]-GTP-loaded GST-Rac-1 (400 nM) was examined by a filter-binding assay. The concentration of the positive control GST-SptP(70–544) in this reaction was 40 nM (values represent the results of one of three experiments that gave equivalent results, with a standard deviation of less than 5%). **c**, The GAP activity of SptP is required for the recovery of the actin cytoskeleton after bacterial infection. Ref52 cells were infected with wild-type *S. Typhimurium*, the isogenic ΔsptP strain, the isogenic ΔsptP carrying a plasmid encoding either wild-type SptP (pSptP) or the GAP-deficient mutant SptP(R209A) (pSptP^{R209A}), and 3 h after infection the integrity of the actin cytoskeleton in infected cells was examined by fluorescence microscopy (see legend to Fig. 1). The results are the mean and standard deviations of three independent experiments.

constructed by site-directed mutagenesis using PCR. Plasmids pSB759 and pSB1473, which express wild-type SptP and the GAP-defective mutant SptP^{R209A}, respectively, from an arabinose-inducible promoter were constructed by standard recombinant-DNA techniques.

Cell lines, bacterial infections, microinjection of cultured cells and immunofluorescence microscopy

Ref52 cells were used as they possess a well developed actin cytoskeleton. Microinjection of these cells, bacterial infections, antibody staining and fluorescence microscopy were all carried out as described⁹.

GST pull-down assay

GST–Rac1 or GST–Rac-1^{L61} purified as described³, were bound to glutathione-4B agarose beads in 100 µl PBS buffer at 4 °C for 30 min. Beads were then washed with binding buffer (20 mM phosphate buffer, pH 7.4, containing 1% Triton-X100, 1 mM DTT) before being added to a lysate of *E. coli* DH5α carrying plasmid pSB759 or pSB1473, which expresses SptP or SptP^{R209A} under an arabinose-inducible promoter, or to a solution of purified SptP protein cleaved from the GST moiety by thrombin (data not shown). Bacterial lysates were prepared by sonication in binding buffer after growth under inducing conditions (growth for 5 h in LB containing 0.005% arabinose). Cleavage of SptP from the GST moiety was carried out as described⁵. The beads prebound to GST-fusion proteins were incubated with the bacterial lysates or purified SptP protein at 4 °C for 30 min and washed three times in binding buffer. The bound proteins were eluted in sample buffer and analysed by standard western blotting using a monoclonal antibody against SptP.

JNK activation assay

Detection of the phosphorylated (active) form of JNK was carried out by western blotting with a phosphospecific antibody according to the manufacturer's instructions (New England Biolabs). Quantification of phosphorylated JNK was carried out by densitometric analysis of films exposed in the linear intensity range. Polyclonal antibody against JNK was from Santa Cruz Biotechnology.

GAP assays

Purified small GTP-binding proteins fused to GST were loaded with 50 nM of GTP (1:30 ratio of [α -³²P] GTP to cold GTP) in 20 µl GTP-loading buffer (20 mM Tris, pH 8.0, 2 mM EDTA, 1 mM DTT) at room temperature for 15 min. GTP hydrolysis was initiated upon addition of MgCl₂ and different forms of SptP or control protein, as indicated. After 15 min, GTP and GDP were eluted from the samples by 0.4% SDS, 5 mM EDTA, 5 mM GTP and 5 mM GDP at 65 °C for 5 min and separated by thin-layer chromatography on polyethyleneimine–cellulose sheets (Scientific) with 0.75 M KH₂PO₄, pH 3.5, as the developing solvent, followed by autoradiography and PhosphorImager quantification (Molecular Dynamics). Alternatively, GAP activity was measured by a filter-binding assay²¹. All filter-binding assays were carried out using GST–SptP^{70–544}, which lacks the secretion and translocation signals and is more soluble than GST–SptP^{1–544}.

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Deregulated cyclin E induces chromosome instability

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Cyclin E, a regulatory subunit of cyclin-dependent kinase 2 (Cdk2), is an important regulator of entry into S phase in the mammalian cell cycle. In normal dividing cells, cyclin E accumulates at the G₁/S-phase boundary and is degraded as cells progress through S phase^{1,2}. However, in many human tumours cyclin E is overexpressed³ and the levels of protein and kinase activity are often deregulated relative to the cell cycle^{4–7}. It is not understood how alterations in expression of cyclin E contribute to tumorigenesis. Here we show that constitutive cyclin-E overexpression in both immortalized rat embryo fibroblasts and human breast epithelial cells results in chromosome instability (CIN). In contrast, analogous expression of cyclin D1 or A does not increase the frequency of CIN. Cyclin-E-expressing cells that exhibit CIN have normal centrosome numbers. However, constitutive overexpression of cyclin E impairs S-phase progression, indicating that aberrant regulation of this process may be responsible for the CIN observed. These results indicate that downregulation of cyclin-E/Cdk2 kinase activity following the G₁/S-phase transition may be necessary for the maintenance of karyotypic stability.

There is considerable evidence that alteration of expression of cyclin E contributes to the development of many types of human tumours. Overexpression of cyclin E correlates with advanced grades and stages of tumour and has prognostic significance in many tumour types^{8–11}. Expression of cyclin E is elevated in premalignant lesions of the breast and skin, indicating an early event in the development of these tumours^{8,11}. Additionally, constitutive overexpression of cyclin E specifically targeted to the mammary epithelium of transgenic mice increases the incidence of breast hyperplasias and carcinomas¹².

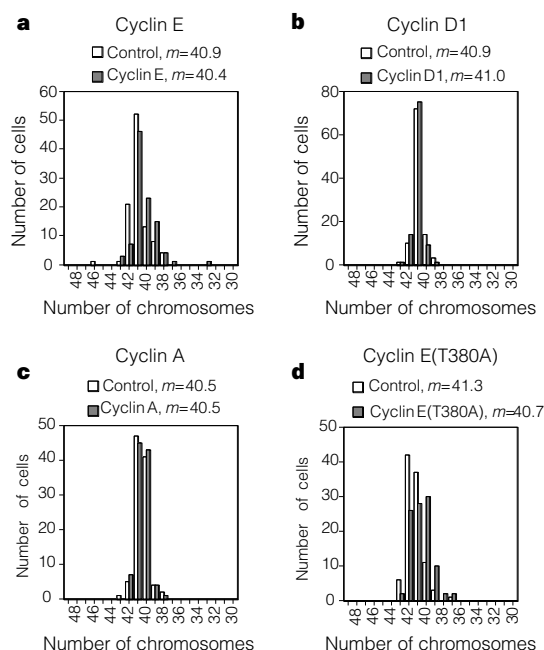


Figure 1 Chromosomal distributions of REFs capable of the conditional deregulated overexpression of various human cyclins. Cell lines expressing **a**, cyclin E, **b**, cyclin D1, **c**, cyclin A or **d**, a hyperstabilized cyclin E(T380A) mutant were passaged in medium that allowed either the repression (control) or expression of the cyclin transgene. Cells were treated with nocodazole and colcemid and karyotypic analysis was performed on 100 metaphase spreads for each experiment. *m*, mean.

Appropriate regulation of cyclin E is critical for a number of cellular processes that could influence the fidelity of chromosome transmission, including the licensing of origins of DNA replication^{13,14} and centrosome duplication^{15,16}. Therefore, we tested whether constitutive overexpression of cyclin E, as observed in many tumours, can affect chromosome stability. Immortalized rat embryo fibroblasts (REFs) that can conditionally overexpress human cyclins through the tetracycline (tet)/tTA gene-induction system were used¹⁷. Induction of the cyclin E transgene in these cells results in constitutive overexpression of cyclin E and an approximately fourfold increase in associated H1 kinase activity¹⁸, comparable to that observed in tumours that overexpress cyclin E⁴.

Following induction or repression of the cyclin E transgene during a 30-day growth period, cells were treated with the microtubule-disrupting agents nocodazole and colcemid and subjected to karyotypic analysis. There was a significant difference between the chromosomal distributions of the induced and control cell populations (Fig. 1a). Two independently derived REF cell lines that overexpressed cyclin E constitutively accumulated an increased proportion of aneuploid cells compared with control cell populations, which remained unchanged. Comparison of the mean total chromosome numbers showed that cyclin-E-expressing cells contained 0.3 to 0.5 fewer chromosomes per cell than control cells (Table 1). We also included in our analysis cells that expressed a mutated form of cyclin E that lacks a Cdk phosphorylation site required for targeting the protein for ubiquitin-mediated proteolysis¹⁹. The cyclin E(T380A) mutant protein is hyperstable in REFs and results in an ~10-fold increase in associated kinase activity. Deregulated overexpression of cyclin E(T380A) in REFs induced CIN at a frequency even higher than that observed in cells expressing wild-type cyclin E (Fig. 1d). Cells expressing cyclin E(T380A) contained 0.4 to 0.6 fewer chromosomes per cell than control cells (Table 1). In contrast, constitutive overexpression of

Table 1 Chromosome instability in cyclin-E-expressing cells

Cell line/cyclin	Experiment	Expression	Mean no. of chromosomes	Chromosome loss/gain (per cell)	Confidence level*
E	1.1	–	40.9		
		+	40.4	–0.5	>99%
	1.2	–	40.8		
		+	40.4	–0.4	
	1.3	–	41.0		
		+	40.6	–0.4	98%
	2.1	–	40.9		
		+	40.4	–0.5	
	2.2	–	40.7		
		+	40.4	–0.3	
	2.3	–	41.0		
		+	40.6	–0.4	
E(T380A)	3.1	–	41.3		
		+	40.7	–0.6	99%
	3.2	–	41.2		
		+	40.8	–0.4	
	3.3	–	41.4		
		+	41.0	–0.4	
A	1.1	–	40.5		
		+	40.5	0.0	id†
	1.2	–	40.5		
		+	40.5	0.0	
	1.3	–	40.5		
		+	40.5	0.0	
D1	1.1	–	40.9		
		+	41.0	+0.1	<90%
	1.2	–	40.9		
		+	40.9	0.0	
	1.3	–	40.9		
		+	41.0	+0.1	

* Confidence level determined using Student's *t* distribution.

† id, indeterminate.

either cyclin D1 or cyclin A had no effect on the chromosomal distribution (Fig. 1b, c; Table 1).

Next we determined whether the CIN observed in cyclin-E-expressing REF cells correlated with alterations in the transgene-induction system. Populations of control cells and cells expressing cyclin E and cyclin E(T380A) were identical with respect to retention of the integrated transgene plasmids, the percentage of cells capable of expressing cyclin E protein and the amount of cyclin E protein expressed (data not shown). Therefore, the observed CIN was not related to changes in transgene expression or selection of differentially expressing clones. In addition, constitutive overexpression of wild-type cyclin E did not alter cell growth rate, making it unlikely that the observed CIN could result from selecting against poor growth (data not shown).

We carried out a more detailed karyotypic analysis to determine whether the observed CIN was due to specific or general chromosome alterations. REF chromosomes were divided into three categories based on the position of the centromere: acrocentric, metacentric or telocentric. Constitutive overexpression of cyclin E was found to induce CIN in each of the three subtypes (Fig. 2a). CIN within each subtype consisted of chromosome losses as well as gains, indicating that cyclin E conferred a general chromosomal instability and that clonal selection due to loss or gain of specific chromosomes did not occur during culturing. Furthermore, CIN was observed in chromosome subtypes in cells that expressed cyclin E and had no net change in total chromosome number (that is, loss within one subtype was countered by a gain within another), suggesting that the extent of instability indicated by total chromosome numbers may be an underestimate.

Cyclin E is expressed at elevated levels in a significant proportion of breast tumours, where it is associated with increased mortality^{6–8}. Furthermore, the subset of breast tumours that exhibit elevated expression of cyclin E and low levels of cell proliferation, that is,

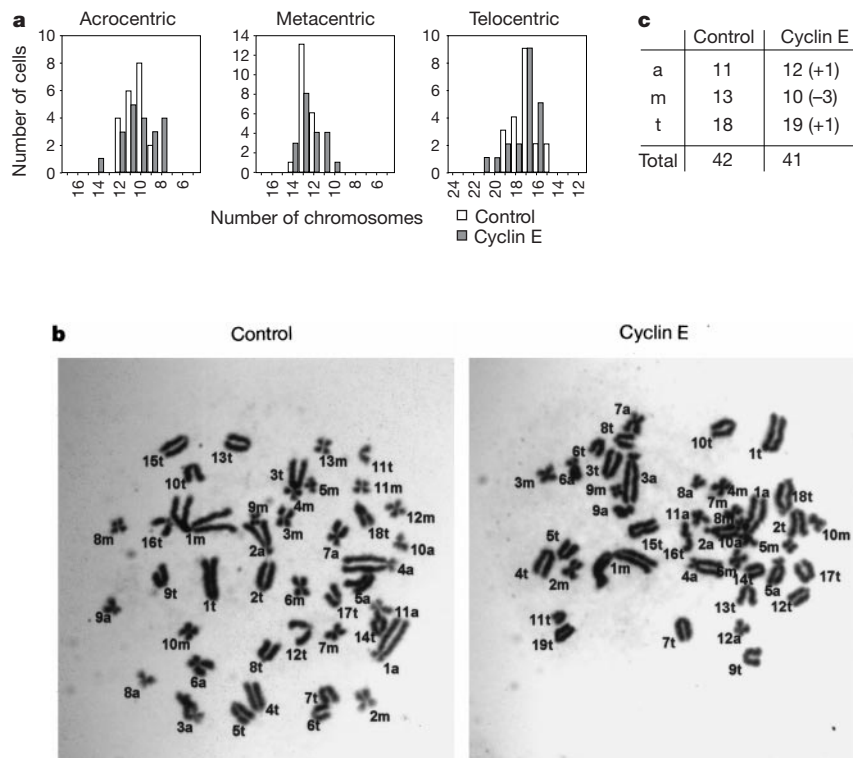


Figure 2 More detailed karyotypic analysis of cyclin-E-expressing cells. **a**, Distribution of chromosome subtypes following cyclin-E expression. **b**, example of karyotypic analysis on

metaphase spreads for a control and a cyclin E-expressing cell and **c**, quantification of the different chromosome subtypes. a, acrocentric; m, metacentric; t, telocentric.

tumours that deregulate expression of cyclin E relative to the cell cycle, are also associated with poor prognosis^{6,7}. Therefore, we next tested whether constitutive expression of cyclin E(T380A) could also induce CIN in immortalized human breast epithelial cells. Following induction or repression of the cyclin E(T380A) transgene, CIN was measured by fluorescence *in situ* hybridization (FISH) analysis using three randomly selected chromosomal markers. The proportion of cells that were aneuploid for each chromosome marker (both chromosome loss and gain) was higher in cells expressing cyclin E(T380A) than in control cells (Fig. 3). Statistical analysis revealed that the aneuploidy generated by cyclin E expression, as reflected by an increase in the variance of the distribution for each marker, was highly significant ($P < 0.005$) between the control and cyclin-E(T380A)-expressing cell populations (Fig. 3). Increases in the frequency of chromosome loss as well as gain for each of the three chromosomes indicated again that the observed CIN could not be due to clonal selection. Although the CIN for each individual chromosome was modest, these data indicate that the combined CIN for the entire karyotype may be high.

CIN could result from alterations in a number of cell division processes²⁰. Constitutively elevated expression of cyclin E and associated kinase activity could induce CIN by phosphorylating targets at inappropriate times in the cell cycle, possibly causing misregulation of those processes critical to maintaining the fidelity of chromosome transmission. Therefore, we expressed cyclin E constitutively at various elevated levels in REFs to find out which cell-cycle processes might be adversely affected by inappropriate cyclin-E/Cdk2-associated kinase activity. Constitutive overexpression of cyclin E, and to a greater extent cyclin E(T380A), in our REF cell lines impeded progression of cells through S-phase (data not shown). We also infected R12 cells, the parental line from which the cyclin-expressing cell lines were derived, with a recombinant adenovirus that allowed overexpression of cyclin E at significantly higher levels than the tet/tTA gene-induction system. A dose-

dependent increase in the percentage of S-phase cells and decrease in cell proliferation was observed with increasing multiplicity of infection (MOI) of the cyclin E adenovirus. These results indicate that constitutive overexpression of cyclin E induces S-phase delay or arrest and suggest that impairment of chromosome replication may be linked to the generation of CIN.

Cyclin-E/Cdk2 activity is required for repeated centrosome duplication in S-phase-arrested *Xenopus* egg extracts^{15,16}. Deregulated cyclin-E/Cdk2 kinase activity could presumably induce CIN by altering centrosome number and causing the formation of multipolar mitotic spindles, a phenotype that has been observed in human tumours^{21,22}. We next tested for centrosome abnormalities by staining for γ -tubulin in the cyclin-expressing REF cell lines used in our experiments. We found no increase in the frequency of cells exhibiting abnormal numbers of centrosomes in those cyclin-E- and cyclin-E(T380A)-expressing cells that exhibited CIN, or in cells expressing cyclin D1 or A, compared with control cells (data not shown). Therefore, centrosome abnormalities are unlikely to be the predominant mechanism by which cyclin E and cyclin E(T380A) induce CIN in the REF cell lines used.

We can only speculate on how deregulated overexpression of cyclin E leads to CIN. Adenovirus-infected S-phase R12 cells were found to show significantly less BrdU incorporation than cells infected with control virus, consistent with a reduced rate of DNA replication (data not shown). The S-phase delay and reduced rate of DNA synthesis caused by elevated cyclin E could be linked to the proposed role of cyclin E in licensing origins of DNA replication. Deregulated cyclin-E/Cdk2 kinase activity at the M/G₁ boundary in *Xenopus* egg extracts has been shown to prevent the association of minichromosome maintenance (MCM) proteins with chromatin, thereby interfering with the efficient establishment of pre-replication complexes^{13,14}. Furthermore, reduced MCM protein function in budding yeast causes chromosome instability²³. Thus, failure to license chromosomal origins of

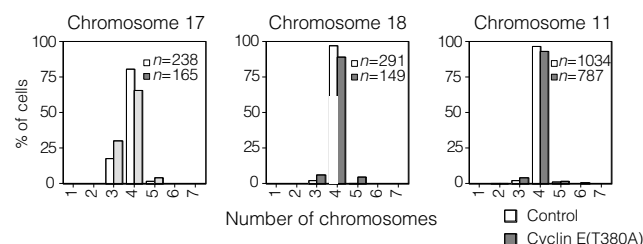


Figure 3 Cyclin E(T380A) induced CIN in human breast epithelial cells. Following repression (control) or expression of the transgene, the number of copies of chromosomes 11, 17 and 18 was determined by FISH analysis. Results show combined data from at least two independent experiments. Note that this cell line is tetraploid with four copies of each chromosome per cell. Also, chromosome 17 appears to have undergone a significant level of CIN before the experiment. The increase in the variance of the cyclin-E(T380A)-expressing cell distribution relative to the control cell distribution for each chromosome marker was highly significant ($P < 0.005$) as determined by the cumulative F test²⁰.

Table 2 Induction of polyploidy in cyclin-E-expressing cells

Cell line/cyclin	Expression	% Polyploid cells*
E	–	1.2
	+	2.4
	–	1.3
	+	3.3
E(T380A)	–	0.2
	+	1.2
A	–	0.7
	+	0.6
D1	–	2.8
	+	2.3

* Each value reflects the average of two independent experiments involving the analysis of ≥ 750 cells each.

replication efficiently could lead to impairment of S-phase and ultimately to CIN.

Our results show that deregulation of cyclin-E/Cdk2 kinase activity can affect the fidelity of chromosome transmission in mammalian cells. These results underscore the importance of alterations in cyclin E as an early event in the genesis of some tumours, and its involvement in tumour progression, driving cells towards more advanced tumour stages by unmasking recessive mutations in tumour suppressor genes. We have found that constitutive overexpression of cyclin E does not significantly increase the frequency of gene amplification (another form of genetic instability; data not shown), but does cause a modest increase in the frequency of polyploid (> 2 chromosome DNA content) cells, as described previously^{24,25} (Table 2). These data indicate that alteration of cyclin-E/Cdk2 kinase activity may not influence the frequency of all forms of genetic instability, but may specifically affect those processes involved in the faithful duplication and segregation of chromosomes. □

Methods

Cell lines and culture

REF derived (Rat-1) cell lines (A8, D5, E12, E18 and T13.11) capable of the inducible expression of human cyclin A, D1, E or E(T380A), respectively, and parental cell line R12 that lacks a cyclin expression plasmid, as well as conditions for cell culturing and procedures used in cell-cycle analysis, have been described^{18–19,26}. The REF cell lines contain a quasi-diploid (2C) DNA content and are wild-type for both p53 and pRb¹⁹. The human breast epithelial cell line capable of conditional expression of cyclin E(T380A) was derived from cell line 184A1-1 and contains a quasi-tetraploid (4C) DNA content. The tetracycline regulatory elements are contained on two integrated plasmids—a tet transactivator (tTA) sequence and a tet operator (tOp) sequence linked to the cytomegalovirus (CMV) minimal promoter. For deregulated cyclin overexpression experiments, 1×10^6 starting cells were passaged at sub-confluent levels for 30 days in culture medium supplemented with (control) or without tetracycline (cyclin expression).

Karyotype and FISH analyses.

Cell cultures were incubated in medium containing nocodazole ($0.1 \mu\text{g ml}^{-1}$) and colcemid ($0.1 \mu\text{g ml}^{-1}$) for 4 h. Mitotic cells were isolated, incubated in hypotonic medium (diluted 1:1 with dH_2O) and fixed with methanol, and the chromosomes were stained with Giemsa (Sigma). Chromosomal distributions included the analysis of 100 metaphase spreads for each experiment, excluding polyploid cells. We determined the percentage of polyploid cells by analysing at least 750 metaphase spreads for selected experiments. More detailed karyotypic analysis was performed on 20 metaphase spreads for both control and cyclin-E-expressing cells. FISH analysis was performed on cells using centromeric probes specific for chromosomes 11, 17 and 18 as described by the manufacturer (Vysis).

Centrosome staining.

Centrosomes were analysed in REF cell lines following 4, 8 or 30 days of cyclin transgene expression or repression. Cell fixation and staining for γ -tubulin were as described²⁷. At least 500 cells were analysed for each experiment.

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The thymine glycosylase MBD4 can bind to the product of deamination at methylated CpG sites

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In addition to its well-documented effects on gene silencing, cytosine methylation is a prominent cause of mutations. In humans, the mutation rate from 5-methylcytosine (m^5C) to thymine (T) is 10–50-fold higher^{1–4} than other transitions and the methylated sequence CpG is consequently under-represented⁵. Over one-third of germline point mutations associated with human genetic disease⁶ and many somatic mutations leading to cancer^{7,8} involve loss of CpG. The primary cause of mutability appears to be hydrolytic deamination. Cytosine deamination produces mismatched uracil (U), which can be removed by uracil glycosylase^{9,10}, whereas m^5C deamination generates a G-T mismatch that cannot be processed by this enzyme. Correction of m^5CpG -TpG mismatches may instead be initiated by the thymine DNA glycosylase, TDG^{11,12}. Here we show that MBD4, an unrelated mammalian protein that contains a methyl-CpG binding domain^{13,14}, can also efficiently remove thymine or uracil from a mismatches CpG site *in vitro*. Furthermore, the methyl-CpG binding domain of MBD4 binds preferentially to m^5CpG -TpG mismatches—the primary product of deamination at methyl-CpG. The combined specificities of binding and catalysis indicate that this enzyme may function to minimize mutation at methyl-CpG.

MBD4 (ref. 14) was identified in a database search for proteins with a methyl-CpG-binding domain (MBD) resembling that of the transcriptional repressor MeCP2 (refs 13, 15). Hypothetical translation of the full-length human and mouse MBD4 complementary DNAs revealed an amino-terminal methyl-CpG-binding domain

and a carboxy-terminal region that was closely related to the glycosylase/endonuclease domains of bacterial repair proteins (Fig. 1a). Related proteins include the 8-oxoG-A mispair-specific adenine glycosylase MutY of *Escherichia coli*¹⁶, the G-T mismatch-specific thymine glycosylase Mig of *Methanobacterium thermoautotrophicum*¹⁷, the thymine glycol glycosylase EndoIII of *E. coli*¹⁸ and the photodimer-specific UV-endonuclease of *Micrococcus luteus*¹⁹ (Fig. 1b). Mouse and human MBD4 proteins are 86% and 95% identical in the methyl-CpG-binding domain and the C-terminal glycosylase-like domain, respectively, but are less conserved elsewhere (Fig. 1a).

On the basis of these relationships, we asked whether human MBD4 has DNA glycosylase or endonuclease activity. The presence of a methyl-CpG-binding domain led us to postulate that MBD4 might be involved in the processing of DNA damage associated with methylated CpG sites. Upon incubation of recombinant human MBD4 with a fluorescently labelled oligonucleotide duplex containing a single G-T mismatch in a CpG context, an abasic site was generated by the removal of the mispaired pyrimidine by glycosidic bond cleavage (Fig. 2a). In addition to the G-T mispair, G-U substrates were also cleaved (Fig. 2b), but the processing of other base/base mismatches, including C-A, C-C and G-G, was not observed (data not shown). That MBD4 is a mismatch specific T/U DNA glycosylase was confirmed by high-performance liquid chromatography (HPLC) analysis of the product mixture. Free T or U could be detected in reaction mixtures which contained MBD4 and G-T or G-U mismatches, respectively (data not shown). The sugar-phosphate backbone of DNA was not cleaved unless treated with hot alkali or the human AP-endonuclease HAP1 (ref. 20; and Fig. 2a). HAP1 cleaves oligonucleotide substrates at the 5' end of abasic sites. In the assay shown in Fig. 2a, HAP1 generates two fragments: a 5' fluorescein-labelled 23-mer terminated at its 3' end with a hydroxy group; and a 37-mer that has at its 5' end the baseless sugar phosphate from which the mispaired T has been removed by the action of MBD4. Neither glycosylase nor endonuclease activity could be detected on perfectly matched substrates containing symmetrically methylated, hemimethylated or non-methylated CpG sites (Fig. 2b). From these experiments, we conclude that MBD4 is a mismatch-specific T/U glycosylase that lacks endonuclease activity.

A CpG sequence context for the mismatch was preferred, but was not absolutely required, as G-T and G-U mismatches in other sequences were also processed, albeit at a reduced rate (Fig. 2c;

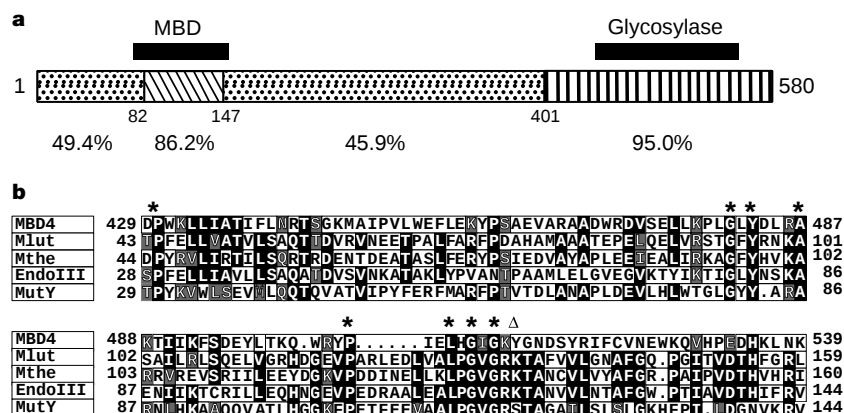


Figure 1 MBD4 contains a conserved glycosylase-like domain and a methyl-CpG-binding domain. **a**, Diagram of MBD4 showing the N-terminal MBD and the C-terminal glycosylase domain. Percentage conservation between human and mouse proteins is shown below each shaded domain. **b**, Amino-acid sequence alignment of human MBD4 (amino acids 429–539) with the *E. coli* thymine glycol glycosylase EndoIII, the 8-oxoG-A-

specific adenine glycosylase MutY, the Mig G-T glycosylase of *M. thermoautotrophicum* (Mthe) and the *M. luteus* UV endonuclease (Mlut). Asterisks mark residues found in all the proteins; triangle indicates the residue that, when basic (K), is diagnostic of an endonuclease (glycosylase/lyase).

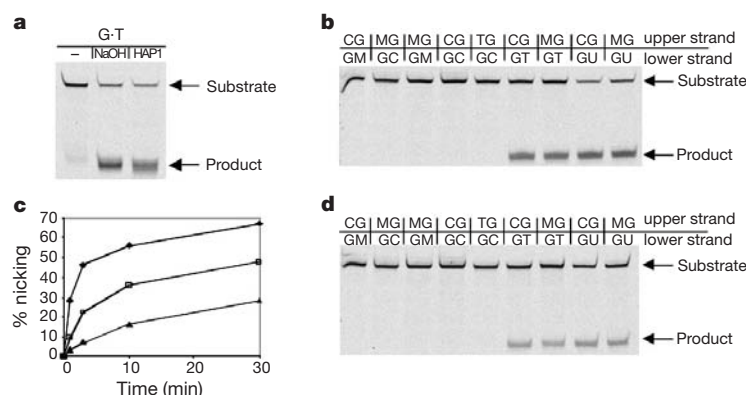


Figure 2 MBD4 is a mismatch-specific T/U DNA glycosylase. **a**, MBD4 does not cleave the sugar-phosphate backbone of a mismatched DNA substrate (lane -). Cleavage occurs only upon the addition of hot alkali (lane NaOH) or human HAP1 endonuclease (lane HAP1). **b**, G-T and G-U mismatches are substrates for T glycosylase processing by full-length MBD4. Oligonucleotide substrate JJ, containing the appropriate mismatches or the corresponding perfectly matched, methylated or unmethylated controls, was incubated with MBD4 (see Methods). Apyrimidinic sites were then cleaved with hot alkali. Substrate

and product bands are indicated. **c**, Kinetics of MBD4 glycosylase action in CG and non-CG sequence contexts. G-U (diamonds) or G-T (squares) in a CG context is compared with G-T in a GG context (triangles). **d**, A C-terminal fragment of MBD4 that includes the glycosylase domain (amino acids 379–580; see Fig. 4a) can process G-U or G-T mismatches. **a**, **b** and **d** are fluorescence scans of denaturing 15% polyacrylamide gels. M denotes m°C.

and data not shown). The methylation status of cytosine in CpG-TpG or CpG-UpG mismatches did not affect the glycosylase reaction in this assay (Fig. 2b). Substrate specificity *in vitro* is therefore similar to that of the other known mammalian G-T mismatch-specific DNA glycosylase, TDG^{11,12}. As anticipated from the amino-acid sequence alignment (Fig. 1b), the glycosylase activity resides in the C-terminal region of MBD4, as a polypeptide corresponding to amino acids 379–580 could process G-T and G-U mismatches (Fig. 2d), whereas polypeptides in which the C terminus was deleted were inactive (data from Figs 2 and 3 are summarized in Fig. 4a; and data not shown). The substrate specificity and flanking sequence preference of full-length MBD4 and its C-terminal fragments were similar (compare Fig. 2b, d), indicating that the methyl-CpG-binding domain (see below) did not influence site preference in this assay.

Next, we investigated the DNA-binding specificity of MBD4. Mouse MBD4 binds to densely methylated DNA molecules *in vitro* and localizes to heavily methylated foci in mouse cell nuclei when overexpressed¹⁴. Bandshift assays with probes that contained non-base-paired regions, however, revealed additional specificity. Human MBD4 gave a strong complex with a probe that had three m⁵CpG-TpG mismatches but bound relatively weakly when m⁵CpG-m⁵CpG pairs were substituted at these sites (Fig. 3a, left panel). The probe with non-methylated, mismatched CpG-TpG sites also gave a weak complex. We conclude that both G-T mismatch and m⁵C moieties are important for recognition by MBD4. Dependence of mismatch binding on the methyl-CpG-binding domain was shown by bandshift assays with truncated versions of MBD4. The N-terminal 165 residues, which include the DNA-binding domain, retained specificity for the methylated mismatch (Fig. 3a, right panel), whereas fragments that excluded all or part of the domain could not bind any of the probes (Fig. 4a; polypeptides 1–580 and 379–580). The results show that MBD4 preferentially binds to the mismatch that results from hydrolytic deamination of m⁵C in mammalian genomic DNA.

The complexes in Fig. 3a were formed under conditions in which processing of the mismatched site is undetectable (data not shown). After incubation for 20 min at 37°C, MBD4–DNA complexes reproduced the specificity of the glycosylase reaction, which prefers mismatches in a CpG context, but is indifferent to methylation (Fig. 2b). The full-length protein and a C-terminal fragment lacking

the methyl-CpG binding domain (379–580; Fig. 3c) formed a complex with each of the mismatched probes (Fig. 3b), but a truncation lacking the C-terminal glycosylase-like domain did not form the complex (data not shown). As other DNA glycosylases bind tightly to the abasic site resulting from their catalytic action²¹, we suspected that the observed complexes also represented product binding by MBD4. Accordingly, we found that the glycosylase domain (379–580) and the intact form of MBD4 bound efficiently to abasic sites under the same conditions (Fig. 3c; and data not shown). Thus the N terminus of MBD4 binds to the MG-TG mismatch that is the substrate for base removal, whereas the C terminus, following processing, binds to the abasic product of the reaction.

MBD4 shows no apparent amino-acid sequence similarity to TDG, a protein that also excises mispaired T and U from G-T and G-U mismatches^{11,12}. The two proteins probably evolved from different ancestors, as TDG is related to a bacterial G-U-processing enzyme, Mug²², whereas MBD4 seems to have arisen through the fusion of a glycosylase domain, such as that of bacterial MutY, to an N terminus that may target the enzyme to methylated mismatches. TDG does not contain a methyl-CpG-binding domain. Despite these sequence differences, MBD4 and TDG show very similar catalytic specificities *in vitro*, both enzymes preferring G-T and G-U mismatches within a CpG context²³. In addition, both proteins bind to the apyrimidinic site that arises following mismatch processing (ref. 21; and Fig. 3b, c).

MED1 (identical to MBD4) is reportedly a binding partner of MLH1, a protein implicated in mismatch repair²⁴. It was proposed that MBD4/MED1 is an endonuclease that binds to hemi-methylated CpG sites and directs mismatch repair via a single strand nick towards the newly replicated strand, in a manner analogous to bacterial MutH^{25,26}. Against this hypothesis, we find that MBD4 lacks endonuclease activity. The weak binding of MBD4 to hemi-methylated CpG sites compared with symmetrically methylated CpG²⁴ or m⁵CpG-TpG mismatches also argues against the MutH homologue model.

We propose instead that the function of MBD4 is to counteract the mutability of m⁵C by initiating conversion of m⁵CpG-TpG mismatches back to m⁵CpG-CpG. Among the sequences that we have tested, only m⁵CpG-TpG shows both preferential binding by the N terminus of MBD4 and processing by its catalytic domain

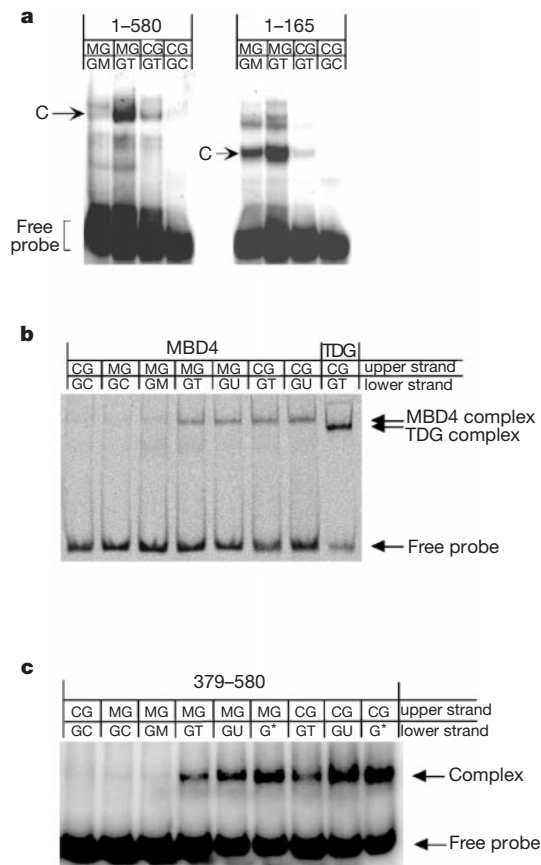


Figure 3 DNA-binding specificities of MBD4 and its truncated derivatives to DNA containing methylated and non-methylated duplexes with or without G-U or G-T mismatches. **a**, Binding of full-length MBD4 (left) and the N-terminal 165 amino acids of MBD4 (right) to probe 'BH' (see Methods), which contains three potential CpG mismatch sites. Within each probe, all three sites were CpG-CpG duplex (CG-CG), m⁵CpG-m⁵CpG duplex (MG-MG), m⁵CpG-TpG mismatch (MG-TG) or CpG-TpG mismatch (CG-TG). Major complexes (C) are indicated. The slower migrating complexes in the MG-MG and MG-TG lanes of the 1-165 panel may be due to binding of more than one protein molecule to the probe. **b**, Full-length MBD4 incubated under conditions that favour the glycosylase reaction with fluorescently labelled probe JJ, in which a single CpG site was methylated or mismatched as shown (see Methods). Complexes were formed with G-U or T-G mismatches regardless of methylation status. For comparison, the complex between CG-TG mismatch and the T glycosylase TDG is shown (lane TDG). **c**, Under the same conditions, a C-terminal fragment (amino acids 379-580; see Fig. 4a) that can carry out the glycosylase reaction complexed with all mismatches in probe JJ, which was mismatched or methylated as shown. Asterisk denotes an abasic site.

(Fig. 4b). Although processing of mismatches *in vitro* was not accelerated by methylation, it is possible that *in vivo* the N-terminal domain targets MBD4 to these sites as they arise by hydrolytic deamination of m⁵C (Fig. 4c). Repair of the resulting abasic site may involve interaction with MLH1 (ref. 24). Given the known importance of CpG to TpG transitions in genetic disease and cancer, it will be of interest to test the effect of MBD4 loss on mutation frequency and cancer predisposition. □

Methods

Protein expression.

MBD4 deletion constructs were made by PCR amplification from an MBD4 cDNA and cloning the resulting products into the pET6H vector. Recombinant proteins were expressed and purified as described¹⁴. N-terminal constructs were made by amplifying with the 5' primer 5'-CCTGCTCCATGGGACGACTGGGCTG-3' and one of the

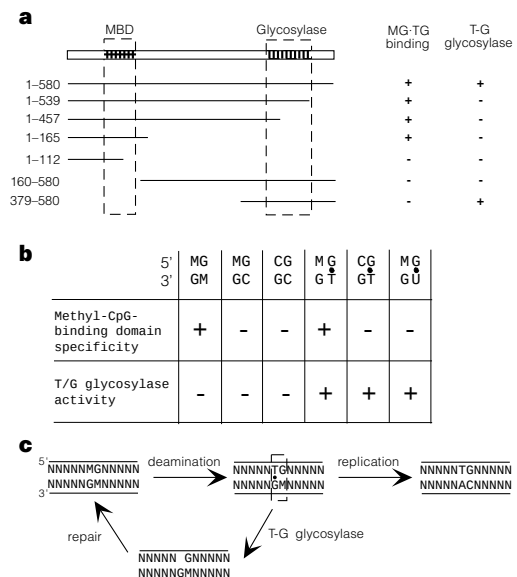


Figure 4 The properties of MBD4 suggest a role in initiating repair of m⁵CpG-TpG mismatches. **a**, Summary of DNA-binding and T glycosylase activity of truncated MBD4 polypeptides against m⁵CpG-TpG (data is from Figs 2 and 3; and data not shown). The failure of the 160-580 protein to process mismatches is unexplained (aberrant protein folding?). **b**, Of the DNA sequences tested, only the methylated mismatch m⁵CpG-TpG is both bound by the N terminus of MBD4 and processed by its glycosylase domain. **c**, A scheme for the role of T-G glycosylases in initiation of repair of deaminated methyl-CpG pairs. Solid circles in **b** and **c** indicate interstrand base mismatches.

following 3' primers: (580) 5'-GCGGGATCCTGAGCTTGAAGCTGCAG-3'; (539) 5'-GCGGGTACCAATCCCATGAAGCTC-3'; (165) 5'-GCGGATCCGATGTC-AGGGCTGCCATG-3'; (112) 5'-GCGCAGCTGATAAAGTACACACTC-3'. The 1-457 construct was made by cloning the N-terminal NcoI fragment of the 1-580 construct into pET6H. C-terminal constructs were made by amplifying with the 580 primer (above) and one of the following 5' primers: (160) 5'-CGCCATGGCAGCCCTGACATC-3'; (379) 5'-GCGCCATGGGCTCTGAAATGGACAACAAC-3'.

Enzymatic activity assay.

The enzymatic activity of the wild-type and mutant recombinant proteins was monitored using a 'nicking assay'. A 60-mer double-stranded oligonucleotide substrate containing different mismatches was prepared by annealing oligonucleotides to give the 'JJ' duplex: 5'-TAGACATTGCCCTCGAGGTACCATGGATCGATGTACCTCAAACTAGACGAATTCGG-3' 3'-ATCTGTAACGGGAGCTCCATGCTACCTAGGCTACAGTGGAGTTTGGATCTGCTTAAGGC-F-5' where F is fluorescein and R = G or A, Y = C, m⁵C, T or U, and Z = C or m⁵C (see underlined dinucleotide). A 0.5 μM solution of the labelled lower strand and 1 μM solution of the unlabelled upper strand were heated for 5 min at 95 °C in 10 mM Tris-HCl pH 8.0, 50 mM NaCl, and the solution was allowed to cool slowly to room temperature. In standard 'nicking reactions', 50 nM substrate and 50 nM protein were incubated for 1 h at 37 °C in a total volume of 20 μl in 1× nicking buffer (50 mM Tris-HCl pH 8.0, 1 mM DTT, 0.1 mg ml⁻¹ BSA, 1 mM EDTA). The reaction was stopped by adding NaOH to a final concentration of 90 mM and heating for 10 min at 99 °C. The NaOH treatment cleaves the AP sites resulting from the removal of the mispaired bases by the glycosylase. After adding 0.5 μl 10 mg ml⁻¹ transfer RNA, 1/10 vol. 3 M NaOAc pH 5.2 and 3 vol. cold ethanol (-20 °C) to the reaction mixture, the DNA was precipitated at -20 °C for 1 h, pelleted by centrifugation and washed with 80% ethanol (-20 °C). The dried DNA was resuspended in 10 μl formamide buffer (90% formamide, 1× TBE) and heated for 5 min at 99 °C before loading on a 15% denaturing polyacrylamide gel. The fluorescein-labelled DNA fragments were detected using the blue fluorescence mode of the PhosphorImager (Storm 860, Applied Biosystems). The HAP1 endonuclease was expressed and purified from a clone donated by I. Hickson.

In some experiments, glycosylase activity was also assayed by incubating 1-10 ng recombinant protein with a radiolabelled 27-mer oligonucleotide,

5'-TCAGATTCCGCGCMGGCTGCGATAAGCT

3'-AGTCTAAGCGCGGTGACGCTATTTCGA

(where M signifies m⁵C), in 25 mM sodium phosphate pH 7.2, 10 mM EDTA, 50 mM NaCl and 1 mg ml⁻¹ BSA for 30 min at 37 °C. Piperidine was added to 1 M and the reaction was incubated at 90 °C for 30 min, after which the reactions were completely dried under vacuum. Samples were resuspended in 90% formamide gel loading buffer²⁷, denatured at 95 °C for 5 min, and separated on a 15% denaturing polyacrylamide gel.

Bandshift assays.

Standard bandshift reactions (Fig. 3a) utilized ³²P-labelled 'BH' probes that contained three CpG sites (underlined below) whose methylation/mismatch status was varied. The BH probes used in Fig. 3c were

5'TCAGATTCGCGCZGGCTGCGATAAGCTGZGCGGATCCZGGGAATTCAGCT3'
3'AGTCTAAGCGCGGYCGACGCTATTGACGYGCCTAGGGYCCCTAAGTCGA5'

where Y = C, m⁵C, T or U, and Z = C or m⁵C (see underlined dinucleotides). The three CpG sites were identically modified/mismatched within each probe. Binding reactions were carried out at room temperature for 30 min in 20 mM HEPES pH 7.9, 25 mM NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA, 4% glycerol, 1% digitonin and 50 ng sonicated *E. coli* DNA. The glycosylase reaction does not occur under these conditions (data not shown). Complexes were electrophoresed through 6% polyacrylamide gels in 0.5 × TBE at 4 °C.

Complexes formed under conditions favourable to the glycosylase reaction (Fig. 3b, c) utilized the fluorescent or ³²P-labelled JJ oligonucleotide (see above) as a probe. In standard gel-retardation reactions, 200 nM protein was incubated with 66 nM labelled oligonucleotide substrate and 333 nM unlabelled homoduplex oligonucleotide in 50 mM Tris-HCl pH 8.0, 1 mM DTT, 5% glycerol, 1 mM EDTA at 37 °C for 20 min. The samples were electrophoresed immediately through a 6% native 0.5 × TBE polyacrylamide gel for 45 min at 100 V. A probe with an abasic site was generated by treatment of oligonucleotide JJ containing a MG-UG mismatch with the enzyme uracil DNA glycosylase.

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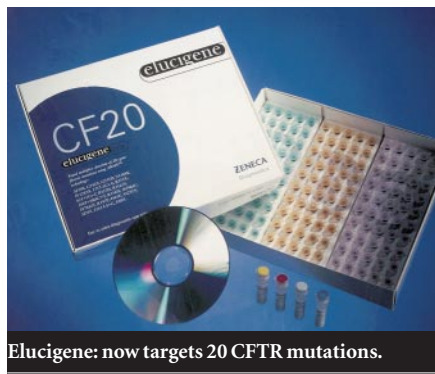
Rustum Roy, Dinesh Agrawal, Jiping Cheng & Shalva Gedevanishvili

Nature **399**, 668–670 (1999)

In the penultimate paragraph of this Letter, there are two instances where “electric field” has been used when “magnetic field” is meant: one is in the fourth sentence and the other in the last. □

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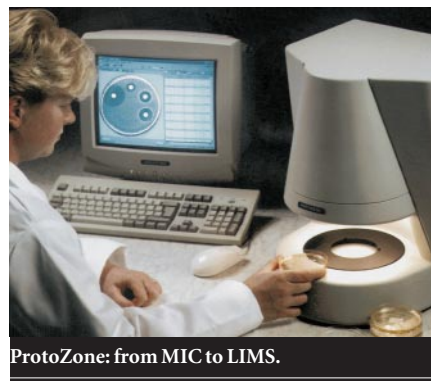
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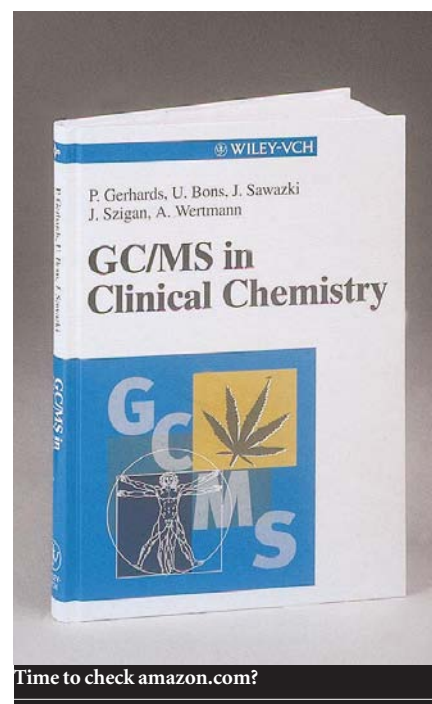
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